Engineered Cx26 Variant Established Functional Heterotypic Cx26/Cx43 and Cx26/Cx40 Gap Junction Channels

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Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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ENGINEERED Cx26 VARIANT ESTABLISHED FUNCTIONAL HETEROTYPIC
Cx26/Cx43 AND Cx26/Cx40 GAP JUNCTION CHANNELS

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by

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Graduate Program in Neuroscience

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science

The School of Graduate and Postdoctoral Studies
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Abstract

Two hexameric connexin hemichannels dock together to form a whole gap junction (GJ) channel. The mechanisms of docking specificity in forming homotypic and heterotypic GJ channels are not fully clear. To reveal the key differences between Cx26 and Cx43 (or Cx40) in their docking residues, we aligned and analyzed ten well studied connexin sequences. Five of them are docking compatible with Cx26 and the rest (including Cx43 and Cx40) are not. According to Cx26 atomic structure at the docking interface, we identified two putative docking residues on the second extracellular domain (E2) that are well conserved within docking compatible connexins, but drastically different between docking incompatible connexins. Switching both of these residues in Cx26 into the corresponding residues in the docking incompatible connexins (K168V-N176H) established morphological and functional heterotypic GJs with Cx43 (or Cx40), indicating these two residues are important for docking incompatibility of these and likely other related connexins.

Key words: Gap junction channel, heterotypic docking compatibility, patch clamp, hydrogen bond
Dedication

I’d like to dedicate this to my family and friends, both here and abroad, who have provided me with continuous support and motivation.
Co-Authorship Statement

Electrophysiological work was accomplished by Levent Berk Karademir, with Honghong Chen providing major assistance in the collection of morphological and localization data for homotypic and heterotypic gap junction combinations. Benny Yue assisted in minor electrophysiological data collection, specifically Cx26K168V-N176H/Cx43 single channel recordings.
Acknowledgments

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### Abbreviations

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>CL</td>
<td>Cytoplasmic loop</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>E1</td>
<td>The first extracellular loop</td>
</tr>
<tr>
<td>E2</td>
<td>The second extracellular loop</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>GJ</td>
<td>Gap junction</td>
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<tr>
<td>Gj</td>
<td>Junctional conductance</td>
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<td>Gj,ini</td>
<td>Initial transjunctional conductance</td>
</tr>
<tr>
<td>HB</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>ICF</td>
<td>Intracellular fluid</td>
</tr>
<tr>
<td>Ij</td>
<td>Macroscopic junctional current</td>
</tr>
<tr>
<td>ij</td>
<td>Junctional current of single channel</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
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<tr>
<td>mV</td>
<td>Millivolts</td>
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N2A Mouse neuroblastoma cells
nS Nanosiemens
NT Amino terminus
pIRES Plasmid containing internal ribosome entry site
pS Picosiemens
RFP Red fluorescent protein
SEM Standard error of the mean
TM Transmembrane domain
V_j Transjunctional voltage
+V_j Positive transjunctional voltage
-V_j Negative transjunctional voltage
V_m Transmembrane voltage
γ_j Unitary channel conductance
Chapter 1 – Introduction

1.1 Gap junction channels

Cellular communication allows for maintenance of homeostasis in tissues and organs in multicellular organisms, and facilitates for quick response by cells to changes in environmental conditions. Gap junctions (GJ), which are clusters of intercellular membrane channels, are key players in cellular communication and serve a crucial role in many physiological processes. The formation of these channels require the membranes of two adjacent cells to be in close proximity of each other, leaving a 2-4 nm gap (Bruzzone et al., 1996). GJs link the cytoplasm of two cells and facilitate the sharing of ions (K\(^+\), Cl\(^-\) and Na\(^+\)), secondary messengers (cAMP), small metabolites (glucose), and small interfering RNAs of up to 1 kDa in size (Loewenstein, 1981; Valiunas et al., 2005).

GJ communication contributes in many crucial processes ranging from development and differentiation, to apoptosis and the maintenance of cell homeostasis (White & Paul, 1999). There can also be detrimental effects due to GJ mediated communication. A dying cell compromised by disease or injury can elicit a “bystander effect”, where GJs allow the passage of metabolites from dying cells to otherwise unaffected healthy neighboring cells. This transfer of substances can lead to the promotion of cell death in the otherwise unaffected cells (Bi et al., 1993). GJs serve another unique purpose in certain cell types. Due to the electrically excitable nature of cells such as neurons, heart and smooth muscles, GJs can allow the propagation of
currents and the electrical synchronization between cell groups (Spray & Burt, 1990). In the brain, GJs facilitate electrical signaling between neurons, and act as a point of passage for metabolites and signaling molecules between glial cells to help support the neurovascular structures (Giaume & Theis, 2010; Pereda, 2014).

There are a number of mechanisms that can modulate and regulate GJs, which can be simply categorized under two major titles – chemical factors and voltage. Chemical factors at play include connexin protein phosphorylation, cytoplasmic pH, intracellular Ca$^{2+}$ concentration, lipophiles, and potentially many more (Harris, 2001). The second category is voltage-dependent deactivation, also referred to as “gating”, which is a common property of all GJs that have been currently identified. Voltage regulation of GJs can be divided into two forms – rectification and transjunctional voltage-dependent gating (Oh & Bargiello, 2015). Rectification is a change in channel conductance due to some form of asymmetry, such as differences in charge distribution in the pore or different post-translational modifications (eg. phosphorylation). GJs formed from different connexins show unique voltage gating characteristics, which might be the reason Cx26/Cx32 heterotypic channels are a good example of rectification (Oh et al., 1999). Voltage-dependent gating can occur due to a number of reasons, such as structural changes in response to voltage, ion availability, Mg$^{2+}$ blocking and distribution of fixed charges in the pore (Oh et al., 2008; Palacios-Prado, Chapuis, et al., 2014). Voltage-dependent structural changes can allow or hinder the flux of ions. Since GJs span the membrane of two adjacent cells, there are two electric fields by which they can be influenced. The first is transjunctional voltage ($V_j$), which is the electrical difference between the interiors of two coupled cells. The second electrical field to consider is the
electrical potential between the cytoplasm and the extracellular space, termed as the membrane potential ($V_m$). Most connexins are only sensitive to $V_j$, with Cx26 being the exception that slightly responds to $V_m$ (Barrio et al., 1991). The molecular origin of $V_j$ gating has yet to be established, however there are two proposed theories. Studies conducted on Cx26 and Cx32 found that changing the charge status of the 2nd amino acid on the N-terminal was sufficient to reverse gating polarity (Verselis et al., 1994), highlighting the NT as a possible origin for $V_j$ gating. Gating polarity is the probability of a given $V_j$ sensitive hemichannel to dwell in the closed state at a relative $V_j$ polarity and intensity (Palacios-Prado, Huetteroth, et al., 2014). Additional research on the Cx32 N-terminal concluded that the first 10 residues of the NT are pore-lining, conferring sensitivity to the $V_j$ field (Oh et al., 2004). The other proposed mechanism highlights a ball and chain model where the C-terminus acts as the gating portion by binding to a receptor position on the cytoplasmic loop, which was a theory drawn from Cx43 and Cx40 studies (Anumonwo et al., 2001). High resolution crystal structure of homomorphic homotypic Cx26 channels support the claim that the NT is in the pore and is in a position to sense differences in $V_j$ (Maeda et al., 2009).
1.2 Connexins

Connexins (Cx) are the basic GJ subunit that oligomerize in a hexamer structure to form half of the GJ channel, also referred to as a hemichannel or connexon. The head to head docking of two hemichannels forms a complete intercellular GJ channel (Harris, 2001). Connexins are seen as unique among other channel proteins due to their functional existence as both a full intercellular GJ channel, and as an undocked hemichannel. Hemichannels have been shown to be important in paracrine signaling (Wang, De Bock, et al., 2013), however are implicated more as pathological rather than physiological entities, based on the current evidence. As observed in the brain and heart, abnormal hemichannel opening can lead to the entry of Na\(^+\) and Ca\(^{2+}\), loss of K\(^+\), ATP and small metabolites, Ca\(^{2+}\) overload, and eventual cell death (Orellana et al., 2014; Wang, De Vuyst, et al., 2013).

There are 21 connexin isoforms in the human genome, and these connexin family members share a similar structural topology. These 21 connexins are placed into one of five categories (α, β, γ, δ and ε) based on their sequence homology (Sohl & Willecke, 2004). Each connexin has four transmembrane domains (TM1-TM4), two extracellular loops (E1 & E2), one cytoplasmic loop (CL), and both amino-terminus (NT) and carboxyl-terminus (CT) on the cytoplasmic side (Milks et al., 1988) (Figure 1.1). The molecular weight of the connexin indicates the nomenclature for the common name of each isoform. For example, the molecular weight of Cx26 is 26kDa (Beyer et al., 1990). Virtually every cell in the body expresses one or more connexins (Saez et al., 2003). This allows for a large variation in hemichannels and gap junction channel composition. The
six oligomerizing subunits of a hemichannel may be formed of the same connexin (homomeric), or from a combination of different connexin isoforms (heteromeric). A complete gap junction channel may be composed of two identical hemichannels (homotypic), or from hemichannels of different composition (heterotypic) (Figure 1.1). Though all these combinations are theoretically possible, not all homomeric channels can dock to form functional heterotypic gap junction channels with one another. Both the E1 and E2 domains are involved in intercellular channel formation, however it was observed that the docking compatibility of two hemichannels was linked to the E2 domain (Bai & Wang, 2014). The mechanisms involved in heterotypic docking compatibility are not fully understood, however it is possible that a small number of differences in the residues of the E2 domain can be responsible for the observed compatibility barrier.
Figure 1.1 Various GJ channel compositions and general topology of a single connexin subunit.

Oligomerization of six connexin subunits forms a hemichannel. Two hemicannels at the plasma membrane can dock to form a full gap junction channel. A hemichannel composed of identical connexins are referred to as homomeric, while those with different connexins are termed heteromeric. Similarly, gap junction channels composed of identical hemicannels are referred to as homotypic and those with different hemicannels are called heterotypic. All connexin isoforms are transmembrane proteins with similar topology.
1.3 Connexin 26

1.3.1 Localization and physiological functions

Human Cx26 (also known as gap junction β-2 protein and encoded by *GJB2*) is an extensively studied connexin, and has been found in almost all locations of the cochlea that form GJ channels (Kikuchi et al., 1995). Cx26 was also shown to be expressed in the skin, specifically during keratinocyte differentiation (Di et al., 2001). Other tissue and cell types that are known to express Cx26 include the liver (Iwai et al., 2000), placenta (Kibschull et al., 2008), and mammary epithelium (Monaghan et al., 1996).

Cx26 mutations are fairly common in the general population. Mutations linked to nonsyndromic hearing loss can be found distributed across the coding region of Cx26, however syndromic mutations that additionally present with skin diseases were found to be located mostly in the N-terminus and E1 domain (Lee & White, 2009). Cx26 mutations causing non-syndromic deafness can be dominant or recessive, with 80% of cases resulting from recessive mutations at more than 100 loci and the remaining 20% caused by dominant mutations on more than 30 loci (Petit et al., 2001; Yan & Liu, 2008). Due to the high frequency of Cx26 mutations, genetic screening for Cx26 is a routine procedure in cases of pediatric hearing impairment (Smith, 2004; Tranebaerg, 2008). Simple loss-of-function mutations in Cx26 have been found to cause nonsyndromic deafness, whereas that is not the case for the syndromic forms (Bruzzone et al., 2003; Zhao et al., 2006). The observation that complete loss of Cx26 function does not result in skin diseases suggests that the human skin does not require Cx26 to maintain homeostasis (Lee & White, 2009). However, the effects of certain Cx26 mutants on the skin in
syndromic hearing loss are apparent, and possibly due to dominant-negative effects on Cx43. Both Cx26 and Cx43 are implicated in skin diseases, and it has been demonstrated that dominant autosomal Cx26 mutants that cause hearing impairment can have trans-dominant inhibition when co-expressed with wildtype Cx43 (Rouan et al., 2001). Another investigation looked into Cx26 knock out (Cx26<sup>-/-</sup>) mouse models to examine its systemic role, however these mice were found to die in utero as a result of placental defects (Gabriel et al., 1998). In order to retain viability, conditional knockouts were found to be a better approach for studying the effects of Cx26 ablation in tissue and system physiology (Stewart et al., 2014)

1.3.2 Determination of crystal structure

The structure of Cx26 was experimentally determined through crystallization of a homomeric homotypic Cx26 GJ channel at a resolution of 3.5 Å (Maeda et al., 2009; Suga et al., 2009). The resolution was high enough to observe the atomic structure and interactions of the Cx26 GJ channel. Some of the major findings were as such: the four transmembrane helices of a connexin were arranged differently than what was proposed previously in a pseudoatomic model, several residues linked to nonsyndromic hearing loss or skin diseases were also involved in inter- or intramolecular interactions, the interactions between the two extracellular domains apposing hemicannels were elucidated in detail, and that the N-terminus restricts the diameter of the pore entrance and might play a role in channel gating (Maeda et al., 2009).

The atomic structure confirmed that both the E1 and E2 domain are involved in mediating docking interactions between two adjoining hemicannels. The two
extracellular domains formed a double-layered channel wall, with the E1-E1 interaction being staggered, possibly to prevent the inner channel from leaking into the extracellular space. An interesting finding was the presence of 60 hydrogen bonds (HB; a non-covalent bond) at the docking interface. The localization of these 60 HBs were divided as 24 HBs being involved in the E1-E1 docking interfaces, and 36 HBs being involved in the E2-E2 docking interfaces between two docked hemichannels. This extensive HB network is believed to be responsible for anchoring the two apposing hemichannels during docking. There is strong evidence suggesting that other closely related connexins might be utilizing the same interaction profile during docking (Bai & Wang, 2014; Nakagawa et al., 2011) (Figure 1.2). The functional necessity of a minimum number of HBs at the E2-E2 interface for functional Cx26/Cx32 channels further supports this theory (Gong et al., 2013).
Figure 1.2 Homology model for Cx26/Cx32 homomeric heterotypic gap junction channel and E2 docking interface interactions.

Homomeric hemichannels of Cx26 (magenta) and Cx32 (green) can dock to form functional heterotypic gap junction channels. The E2 docking site between one pair of docked subunits is enlarged to show docking HBs and the relevant residues at the docking interface. Dotted lines indicate HB formation. Modified from Bai and Wang (2014).
1.4 Connexin 43

Cx43 (also known as gap junction α-1 protein and encoded by GJA1) is the most widely expressed connexin in the human body, and an especially prominent connexin isoform in the heart and brain. It is predominantly expressed in astrocytes and microglial cells in the brain (Giaume & Theis, 2010). Though not present under normal conditions, low level of Cx43 expression was reported in the blood-brain barrier during inflammation (Cronin et al., 2008). In the heart, Cx43 is expressed in the ventrical cardiomyocytes, atria, and endothelial cells (Severs et al., 2008). Cx43 has also been found to control granulosa cell proliferation in the reproductive system, and might be responsible for spermatogenesis in the testis (Kidder & Mhawi, 2002; Roscoe et al., 2001). Other major cell types and organs showing Cx43 expression include the skin and mammary glands (Kelsell et al., 2000; Monaghan et al., 1996). Cx43 is a phosphoprotein with over a dozen phosphorylation sites, which have been shown to be involved in the regulation of channel function and interactions (Lampe & Lau, 2004).

Large numbers of mutations in Cx43 have been linked to the pathological condition of oculodentodigital dysplasia (ODDD) (Paznekas et al., 2009). ODDD affects the whole body and presents with a wide range of symptoms, mainly abnormalities in the eyes, teeth, and fingers (Gorlin et al., 1963). Cx43 deficient (Cx43\textsuperscript{-/-}) mouse models died soon after birth due to right ventricular obstruction tract of the heart (Reaume et al., 1995). It was observed that the postnatal lethality of Cx43\textsuperscript{-/-} could be partially compensated for by the knock-in of Cx32 or Cx40, suggesting that these connexins might share a common function (Plum et al., 2000). However, these Cx32 and Cx40 knock-in mice still showed functional and morphological differences when compared to their
wildtype counterparts. This points toward a certain degree of redundancy that might exist, making it necessary to understand the functional interaction profile of different connexins.
1.5 Heterotypic Gap Junction Docking

Hemichannels extend approximately 20 Å into the extracellular matrix, and are believed to use the facilitation of other membrane-associated molecules to bring apposing membranes into close proximity (Yeager, 1998). Both the E1 and E2 domain have been experimentally proven to be involved in the process of docking (G. Dahl et al., 1994; Maeda et al., 2009). While the molecular specificity of docking is yet to be deciphered, the E2 loop is believed to be the domain responsible. The E1 region has high sequence identity and conserved HB-forming residues among many human connexins (Bai & Wang, 2014). This makes it an unlikely candidate for being involved in the determination of heterotypic compatibility. Furthermore, chimeric connexins with substituted E2 domains showed that the origin of the E2 domain was the determining factor in whether or not two connexins would form functional heterotypic GJ channels (Bruzzone et al., 1994; White et al., 1994). White et al. (1994) observed E2 dependent selectivity when working on Cx50, Cx43 and Cx46 chimeras, while Bruzzone et al. (1994) indicated a similar effect while studying Cx32, Cx38 and Cx43 chimeras.

Connexins have been divided into subgroups (α, β, γ) based on the extent of their sequence similarity and length of the cytoplasmic domain (Kumar & Gilula, 1996). While this system possesses some functional consistency due to its basis on sequence homology, it is not without its shortcomings (Söhl & Willecke, 2009). Going through a large number of functional studies, we can observe the heterotypic and homotypic compatibility of different connexins (Figure 1.3). The nomenclature for connexin subgroups do not effectively account for functional heterotypic compatibility; Cx40 (α subgroup) is heterotypically compatible with some β subgroup connexins (Cx30 and
Cx30.3, but not with others (Cx26 and Cx32). These types of discrepancies prompt the generation of an auxiliary grouping system that takes heterotypic compatibility into consideration. When looking at the functional compatibility of ten of the most extensively studied connexins, a certain pattern can be observed (Bai & Wang, 2014). This pattern allows for the categorization of these ten connexins into two distinct groups – Group 1 and Group 2. Two initial observations can be made between these two groups. Firstly, members of the same group (intra-group) can, in most cases, form functional heterotypic gap junction channels with one another. Secondly, connexins belonging to different groups (inter-group) cannot usually form functional heterotypic GJs with one another. The necessity for such a grouping system is further apparent when we observe that neither Group 1 nor Group 2 consists exclusively of one subgroup of connexins (Figure 1.3); Group 1 has 3 connexins belonging to β subgroup (Cx26, Cx32 and Cx30) and 2 belonging to α subgroup (Cx46 and Cx50), while Group 2 has 3 α subgroup (Cx37, Cx40, and Cx43), 1 β subgroup (Cx30.3), and 1 γ subgroup connexins (Cx45).
Figure 1.3 Heterotypic compatibility of ten selected connexins.

Ten extensively studied connexins are divided into two groups, Group 1 and Group 2, depending on their heterotypic compatibility. As a general rule, members of one group can form functional heterotypic homomeric GJ channels with other intra-group connexins (blue), but not inter-group connexins (orange).
1.5.1 E2-E2 Interactions

E1 and E2 sequence alignment of the ten Group 1 and Group 2 connexins show that both extracellular loops are highly conserved, with the E2 being slightly more variable (Haefliger et al., 1992). The high identity and conservation of the E1 and E2 domain suggests that these regions likely share a similar atomic structure with other members of the connexin family (Bai & Wang, 2014; Nakagawa et al., 2011). A closer look at the E2 sequence of Group 1 and Group 2 members showed an interesting pattern. At a given position in the E2 sequence, most of the residues are either highly variable or highly conserved, except at the 168 and 176 equivalent positions when aligned using Cx26 (Figure 1.4). The 168 and 176 positions, along with the 179 and 177 positions, are important in Cx26 due to their critical role in forming HBs between docking hemichannel (Maeda et al., 2009). The 179 equivalent residues are highly conserved across both Group 1 and Group 2 except for Cx40, while the 177 equivalent residues are highly variable throughout. The high variability at the 177 positions across all ten connexins can be attributed to the fact that the HB was formed on the main peptide chain carbonyl group of the Cx26 threonine residue, thus having no selective pressure for any specific amino acid. The patterns of conservation for positions 177 and 179 make it unlikely that they act as determinants in heterotypic selectivity in docking, leaving positions 168 and 176 as the likely candidates. For Group 1 connexins, asparagine is completely conserved at the 176 equivalent positions. Group 2 connexins, however, express either a histidine (4/5) or tyrosine (1/5) at the same equivalent position. While asparagine contains a carboxamide as a side chain, both histidine and tyrosine contain large aromatics. For the 168 equivalent position, Group 1 connexins hold either a lysine (3/5) or arginine (2/5), both
hosting positively charged and long side chains. Group 2 members express a combination of valine (3/5), alanine (1/5) or threonine (1/5), which are small and non-polar. With respect to the other HB forming positions, This group-specific phenomenon of compatibility formed the basis for our rationale (Bai & Wang, 2014).
**Figure 1.4 Sequence analyses of E1 and E2 domains.**

Sequences were aligned for the E1 and E2 of the same ten connexins previously mentioned (Figure 1.3). Sequence logos were generated for the E2 alignment in order to investigate conservation at docking HB residues. Generated logos were for Group 1 and Group 2 human connexins, as well as cumulative Group 1 and Group 2 connexins from different species. The number of sequences used for the sequence logos were 5, 5, 164, and 169, respectively. Numbering of residues follow that of Cx26. Arrows indicate docking HB residues as determined by the Cx26 atomic structure (Maeda et al., 2009). Bolder arrows point at candidate residues responsible for determining heterotypic compatibility.
1.5.2  E2 Domain in Docking

In order to test the role and influence of HBs at the E2-E2 interface, a number of site-specific Cx26 and Cx32 mutants were studied for their functionality. Based on high sequence identity and putative homology modeling, it was predicted that Cx32 should behave in a structurally similar manner to Cx26 (Gong et al., 2013; Nakagawa et al., 2011). The mutants in question changed the residues at Cx26K168 (to K168V and K168A) and Cx32N175 (to N175H and N175Y). Homology modeling for these mutants predicted a range of alterations to the total number of E2-E2 HBs in a pair of docked E2-E2, from the native six HBs all the way down to zero. By recording the transjunctional conductances ($G_j$) of these mutants in a range of combinations, the influence of HBs at the E2 docking interface was elucidated for Cx26/Cx32 channels (Gong et al., 2013). It was found that a minimum of four HBs between each docked E2-E2 region were necessary to form functional gap junction channels. This study weighs on the importance of HBs at the E2-E2 interface, and their role in facilitating the formation of functional GJs. Group 1 connexins show high sequence identity at their HB forming residues, suggesting that other Group 1 members may also use the mechanisms observed in Cx26. However, this prediction has yet to be experimentally proven. Differences at key residues and the lack of a high-resolution atomic structure for a Group 2 connexins has left us guessing as to the mechanisms at play during their E2-E2 docking.
1.6 Rationale and Hypothesis

Two docking HB forming residues, at positions 168 and 176 of Cx26, are conserved within but divergent between members of Group 1 and Group 2 connexins. A connexin from one group cannot usually form functional GJ channels with a connexin from the apposing group, such as in the case of Cx26 and Cx43. We believe that this is due to differences in the docking domains between Group 1 and Group 2 connexins. We hypothesize that the E2 domain of Cx43 and Cx40 share similar structure and use similar residues as those in Cx26. We further hypothesize that switching the residues at positions 168 and 176 of Cx26 to match the majority residues found in Group 2 connexins (K168V and N176H) will result in forming functional heterotypic GJs between Cx26 and Group 2 connexins, Cx43 and Cx40.

1.7 Objectives

i. Investigate the docking and functional interaction between human Cx26/Cx43 heterotypic gap junction channels, and explore the morphological and functional effects of Cx26 single mutants (K168V and N176H) and double mutant (K168V-N176H) on the formation of functional heterotypic gap junction channels with Cx43. The mutations at K168 and N176 were introduced individually or in combination (single and double mutants) to Cx26. Mutant and wildtype constructs expressing tagged or untagged fluorescent proteins were transfected into mouse neuroblastoma cells (N2A) for subsequent fluorescent imaging for morphology and localization, and dual whole-cell patch clamp analysis for function.
ii. Establish whether the Cx26 variant K168V-N176H can also form functional heterotypic channels with another Group 2 member, Cx40. Similar to above, tagged connexins are used to determine morphology and localization, while a combination of tagged and untagged connexins are utilized in whole-cell patch clamp analysis to determine function.
1.8 References


Engineered Cx26 variant established functional heterotypic Cx26/Cx43 and Cx26/Cx40 gap junction channels.

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2.1 Abstract

Gap junction (GJ) channel mediates direct intercellular communication and is composed of two docked hemichannels, which in turn are connexin oligomers. It is well documented that the docking and formation of functional GJ channels are possible only between docking compatible hemichannels (or connexins). The underlying mechanisms of heterotypic docking compatibility are not fully clear. We aligned the protein sequences of two groups of docking incompatible connexins with that of Cx26, the only connexin with atomic structure information. We found two putative docking residues on the second extracellular domain (E2) that are well conserved within docking compatible connexins, but drastically different between docking incompatible connexins. Switching both of these residues in Cx26 into the corresponding residues in the docking incompatible connexins (K168V-N176H) increased morphological and functional heterotypic GJs with Cx43 (or Cx40), indicating these two residues are important for docking incompatibility of these and likely other related connexins.

Key words: Gap junction channel, heterotypic docking compatibility, patch clamp, hydrogen bond
2.2 Introduction

Communication between cells is crucial in terms of homeostasis and functionality in multicellular organisms. In humans and animals, gap junctions (GJ) are the only direct intercellular channels that mediate cell-to-cell communication between adjacent cells through the exchange of ions, small signaling molecules, and metabolic molecules up to 1kDa in size (Harris, 2001; Kumar & Gilula, 1996; Nicholson, 2003). GJs play an important role in a number of fundamental biological processes (Levin, 2007; Saez et al., 2003; Sarieddine et al., 2009), and as such, mutations in these channels can lead to the development of several inherited diseases, such as hearing loss, cataracts, skin diseases, peripheral and central neuropathy, cardiac arrhythmias and developmental abnormalities (Cottrell & Burt, 2005; Kelsell et al., 2000; Laird, 2010).

Gap junction formation requires the head-to-head docking of hemichannels from neighboring cells, where each hemichannel is composed of a connexin hexamer, also referred to as a connexon (Kumar & Gilula, 1996). Depending on the connexin composition, a hemichannel can be heteromeric or homomeric, while the gap junction may be heterotypic or homotypic. All connexins are believed to share the same topology, consisting of two extracellular loops (E1 and E2), one intracellular loop, and four transmembrane domains, with both the carboxyl and amino terminals in the cytoplasm. Each tissue expresses a unique set of connexins, with the main physiological function of mediating metabolic and electrical synchronization between cells (Saez et al., 2003).

The heterotypic docking interactions between several prominent connexins have previously been charted in *Xenopus* oocyte expression system (Swenson et al., 1989;
Werner et al., 1989; White & Bruzzone, 1996). It has been shown that only compatible connexins can dock together to form functional gap junction channels with one another. The extracellular loops, E1 and E2, are believed to play a critical role in determining docking compatibility, as they are the only domains that exist within the extracellular matrix capable of interacting with other connexin hemichannels. The E1 loop is believed to be involved in the formation of the channel pore and parts of the inner channel wall, whereas the E2 loop is hypothesized to be responsible for determining heterotypic docking specificity (Harris, 2001; Haubrich et al., 1996). The role of the E2 loop in docking specificity is based on studies that used chimeric connexins, where the E2 domain of one connexin was replaced with that of another connexin (Bruzzone et al., 1994; White et al., 1994). In these experiments, chimeric connexins formed functional gap junction channels based exclusively on the origin of the substituted E2 domain. Connexins can be separated into two different docking compatible groups, Group 1 and Group 2, based on their heterotypic docking compatibility (Bai & Wang, 2014). In general, connexins from the same group (intra-group) are able to dock to form functional gap junction channels, whereas those from different groups (inter-group) cannot.

Connexins are highly homologous with respect to their amino acid sequences, especially within the same docking compatible group. The discovery of the high-resolution structure of Cx26 has given us great insight into the molecular interactions found at the docking interface of both the E1 and E2 domains (Maeda et al., 2009). Non-covalent interactions such as hydrogen bonds (HBs) were identified to be prominent interactions during docking between apposing extracellular loop domains (in both E1 and E2). HBs at the E2 docking interface were shown to be critical for heterotypic channel
formation between Cx26 and Cx32, both of which are Group 1 members (Gong et al., 2013; Nakagawa et al., 2011). Alterations of docking HB-forming residues have previously been linked to several diseases, highlighting their importance in connexin function and subsequent human physiology (Akiyama et al., 2007; Alexandrino et al., 2009; Richard et al., 2004). High sequence identity and conservation of these docking HB-forming residues at the E2 domains of Cx26, Cx32 and other docking compatible connexins in Group 1 (Cx30, Cx46 and Cx50) indicate that these connexins may use a similar docking mechanism as reported in Cx26 and Cx32. For Group 2 connexins (mostly docking incompatible to Group 1 connexins), however, the lack of an atomic structure for any of its members has left us guessing as to the mechanism of their docking and if they use the corresponding docking residues at their heterotypic docking interface.

The average of the entire sequence identity for ten Group 1 and Group 2 connexins with that of Cx26 is 51%, and 57% for the E2 domain. With such a high level of sequence identity, it is generally believed that the Cx26 crystal structure is a reliable 3D template for these connexin channels. As such, we hypothesize that Cx43 and Cx40 (members of Group 2 connexins) E2 share similar structure and use similar residues as those in Cx26 (Group 1). Homology modeling and sequence alignment of Cx43, Cx40, and other members of Group 2 connexins places docking HB-forming residue equivalents at positions lining the docking interface, similar to what is observed in Cx26. Sequence alignment indicates two of these putative docking residues at the 168th and 176th positions of Cx26 are conserved within docking compatible connexins but divergent between members of Group 1 and Group 2 (Chapter 1, Figure 1.4). The position and pattern of conservation for these two HB-forming residues suggests they are critical in determining
channel docking specificity and function. To investigate the validity of this statement, the two docking HB-forming residues of Cx26 were mutated to match the equivalent residues found in Group 2 connexins (K168V and N176H). Both single and double mutants of Cx26 were studied for heterotypic docking compatibility with Cx43 or Cx40, however mutating both residues (K168V-N176H) are necessary to form morphological and functional heterotypic GJ channels with Cx43 or Cx40.
2.3 Methods

2.3.1 Construction of Cx26 mutants

Human Cx26 cDNA was obtained by polymerase chain reaction (PCR) and inserted into the pIRES2-EGFP at XhoI and EcoR1 restriction sites to make Cx26-IRES-GFP (untagged). Fusion tagged Cx26 was in frame inserted in pTagRFP-N vector as described earlier (Nakagawa et al., 2011). These untagged and tagged Cx26 constructs were used as a template for the single point mutations, K168V and N176H, as well as the double mutant, K168V-N176H. The single mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following primers:

K168V
Forward: 5' GCAGCGGCTGGTGGTCTGCAACGCCTGG 3'
Reverse: 5' CCAGGCGTTGCAGACCACCAGCCGCTGC 3'

N176H
Forward: 5' TGGCCTTGTCCCCATCTGTGGACTGC 3'
Reverse: 5' GCAGTCCACAGTATGGGACACAGGCCA 3'

The double mutant was generated using two sequential mutagenesis processes with these primers. Human Cx40 and Cx43 cDNA was obtained through PCR and inserted into pIRES2-EGFP and pTagEGFP-N vectors, as described (Sun et al., 2013). All connexin clones were sequenced to confirm the accuracy of the nucleotide sequence.
2.3.2 Cell culture and transient transfections

N2A (mouse neuroblastoma) cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), acquired from Life Technologies (Grand Island, New York, USA). Cells were transferred on 35 mm dishes at 50% confluence to culture overnight. Transfection was performed next day with 0.7 µg of cDNA and 1.4 µl of X-tremeGENE HP DNA Transfection Reagent (Roche Applied Sciences, Indianapolis, IN). Constructs containing the connexin of interest express either tagged or untagged EGFP, RFP or DsRed as reporters. Cells were incubated with transfection reagents for 4 hours, followed by overnight culture in DMEM.

2.3.3 Electrophysiological recordings

Gap junctional coupling and $V_{j}$-gating properties for paired N2A cells expressing connexins with fluorescent protein reporters were assessed using dual whole-cell patch clamp technique as described previously (Bai et al., 2006). For homotypic channel analysis, transfected cells were replated on to 10 mm glass coverslips and left to incubate for 30 minutes to 1 hour prior to patch clamp recording. In the case of heterotypic GJ analysis, RFP or GFP expressing mutant or wildtype cells were detached separately, mixed and co-cultured on glass coverslips for 1-2 hours prior to patch clamp recording. Only cell pairs with one red fluorescent cell and one green fluorescent cell were used for heterotypic channel analysis. Heterotypic designs were incubated longer to ensure that time was not the limiting factor for the formations of functional
channels. Transjunctional conductance \( (G_j) \) was calculated and presented as mean ± SEM. Offline series resistance compensation was used to improve the accuracy of measured \( G_j \) (Musa et al., 2004).

A cover slip with transfected cells was transferred to a recording chamber on an upright microscope (BX51WI, Olympus). Cells were then bathed in extracellular fluid (ECF), which was composed of (in mM): 135 NaCl, 5 KCl, 10 Hapes, 1 MgCl\(_2\), 2 CaCl\(_2\), 1 BaCl\(_2\), 2 CsCl\(_2\), 2 Na Pyruvate, 5 D-glucose, pH 7.2-7.4. Paired cells were patched using two glass micropipettes (pipette resistance 2 – 5 M\( \Omega \)) filled with intracellular fluid (ICF) composed of (in mM): 130 CsCl, 10 EGTA, 0.5 CaCl\(_2\), 3 MgATP, 2 Na\(_2\)ATP, 10 Hapes, pH 7.2. Isolated cell pairs of choice were both voltage clamped at 0 mV. To study transjunctional voltage-dependent gating \( (V_j\text{-gating}) \), one cell in the pair was held at 0 mV while the apposing cell was given voltage steps ranging from ± 20 mV to ± 100 mV in 20 mV increments for a duration of 7 seconds.

The macroscopic transjunctional currents \( (I_j) \) or unitary channel currents \( (i_j) \) were amplified via MultiClamp 700A (Axon Instruments) and then converted to digital signals via an ADDA converter (Digidata 1322A, Molecular Devices, Sunnyvale, CA) and were stored in a PC via pClamp9.2 software. The initial amplitude of \( I_j \)s was measured at each tested \( V_j \) and was used to generate \( I_j - V_j \) plot. Unitary channel currents \( (i_j) \)s were further digitally filtered (low-pass Gaussian filter at 200 Hz) for direct measuring current amplitude. Unitary current \( i_j - V_j \) plot was also constructed for analysis of rectifying properties. Linear regressions \( i_j - V_j \) plot at different range of \( V_j \)s were used to estimate the slope unitary conductance \( (\gamma_j) \).
2.3.4 Data analysis

Data are expressed as means ± SEM. One-way ANOVA followed with Brown-Forsythe's test was used to compare the coupling conductance (G_j) between different homotypic and heterotypic pairs (GraphPad, La Jolla, CA). For consistency, the conductances of all heterotypic Cx26 or its mutant/Cx43 pairs were measured at V_j of -20 mV (on Cx43 expressing cell). Other comparisons and statistical tests used are indicated. Statistical probability of p < 0.05 (*), p < 0.01 (**) or p < 0.001 (***) was used to indicate statistical significance.
2.4 Results

2.4.1 Designing Cx26 variants to establish docking with Cx43 and Cx40

Fig. 1.3 summarizes ten well-studied connexins for their capacity to form functional homotypic and heterotypic GJ channels. Generally connexins within the same docking compatible group, Group 1 or Group 2, are compatible to form functional heterotypic GJ channels, while connexins between these two groups are rarely able to form functional heterotypic GJ channels. Cx26, a member belonging to Group 1, is unable to form heterotypic GJ channels with any member from Group 2. The assignment of each connexin to its appropriate Group was based on conclusions from previous functional studies on these ten connexins. The docking HB-forming residues in the E2 of Cx26 and their equivalent residues showed an interesting pattern between these 2 connexin groups, where 2 out of 4 residues (K168 and N176) were well conserved within a docking compatible group, but not between these two groups. The other 2 residues involved in the docking HB-formation (T177 and D179) in Cx26 either showed a lack of any discernable conservation possibly due to the use of the main chain peptide bond as the HB forming component (T177), or a nearly total conservation (D179). To test our hypothesis of Cx43 and Cx40 using similar residues for docking as those in Cx26, we focused on two putative docking residue differences between the E2 of Cx26 and members of Group 2 connexins, including Cx43 and Cx40, and generated two point variants individually (K168V or N176H) or together, a double mutant (K168V-N176H). K168V was chosen due to the high conservation of lysine (3/5) in the majority of Group 1 members, and an equally high conservation of valine (3/5) in Group 2 members.
N176H was selected due to complete conservation of asparagine (5/5) in Group 1 connexins, and the nearly complete conservation of histidine (4/5) in Group 2 connexins. We predict that these single or double Cx26 mutants will increase the likelihood of forming morphological and functional heterotypic GJ channels with Cx43 or Cx40.
2.4.2 **Homotypic Cx26 single and double mutants show reduced gap junction channel function when compared to wildtype Cx26**

To test if our designed Cx26 mutants are able to be biosynthesized and reach the cell-cell interfaces to form morphological GJs, we individually transfected RFP-tagged wildtype Cx26 and mutants into N2A cells. All single mutants K168V and N176H, and double mutant K168V-N176H were expressed and able to form GJ plaque-like structures at the cell-cell interfaces similar to that observed for wildtype Cx26 (Fig. 2.1A). To determine whether these mutants are capable of forming functional homotypic GJ channels, dual whole-cell patch clamp was used to measure macroscopic transjunctional currents (I_Js) in cell pairs expressing one of these mutants. As shown in Fig. 2.1B and C, majority of cell pairs expressing the single mutants, K168V or N176H, and the double mutant, K168V-N176H, was coupled similar to that of Cx26. However, the average coupling conductance (G_J) of the single mutants was significantly reduced (p < 0.001 in both cases comparing to wildtype Cx26 G_J), while a moderate reduction in G_J was observed for the double mutant (p < 0.05, Fig. 2.1C). The G_J measurements were collected through recordings in cell pairs expressing untagged mutants. Under our experimental conditions, tagged and untagged wildtype Cx26 did not show any detectable differences in G_J (Fig. 2C).
Figure 2.1 Morphological and functional analysis of homotypic Cx26 single and double mutants.

(A) Fluorescent images (left) and their superimposition on DIC images (right) show paired/clustered N2A cells expressing RFP-tagged Cx26, K168V, N176H and K168V-N176H. All homotypic mutant pairs were able to form GJ plaque-like structures (arrows) at the cell-cell interfaces similar to that of Cx26. (B, C) Dual patch clamp recording was used to measure transjunctional current ($I_j$) from N2A cell pairs expressing RFP-tagged and untagged Cx26, K168V, N176H and K168V-N176H. (B) Representative traces of $I_j$ at −20 mV transjunctional voltage ($V_j$) for Cx26, K168V, N176H and K168V-N176H. (C) Bar graph illustrates the transjunctional coupling conductance ($G_j$) of homotypic GJ channels formed in cell pairs expressing untagged Cx26, K168V, N176H, K168V-N176H, as well as RFP-tagged Cx26. Average $G_j$s for tagged and untagged Cx26 were similar. Cell pairs expressing K168V and N176H showed a significant drop in $G_j$ when compared to Cx26 ($p < 0.001$). K168V-N176H cell pairs also showed a significant, yet less prominent, drop in $G_j$ when compared to Cx26 ($p < 0.05$). Homotypic K168V-N176H pairs were also significantly different than the negative control ($p < 0.05$). N2A cells transfected with the empty vector were used to serve as negative controls for these experiments.
2.4.3 Cx26 K168V-N176H, but not L168V or N176H, formed functional heterotypic gap junction channels with Cx43

After determining the characteristics of the Cx26 mutants, we wanted to see if these mutants altered heterotypic compatibility when docked with connexins from the apposing Group. We chose to use Cx43 (Group 2) to heterotypically dock with the Cx26 (Group 1) mutants. In order to establish the morphology and localization of these heterotypic channels, fluorescent images were taken of tagged, heterotypic pairs expressing Cx43, with the Cx26 mutants in N2A cells. Wildtype Cx26/Cx43 pairs showed no GJ plaque formation at the cell-to-cell junction, while the K168V/Cx43, N176H/Cx43, and K168V-N176H/Cx43 pairs formed distinct GJ plaque-like structures (Figure 2.2A). To test if these Cx26 variants, K168V, N176H, and K168V-N176H, are able to form functional heterotypic channels with Cx43, we measured junctional currents (I\textsubscript{j}s) in cell pairs with one expressing Cx26 mutant and the other Cx43. I\textsubscript{j}s recorded at a –20 mV V\textsubscript{j} step showed a marginal increase in amplitude for cell pairs with a single mutant/Cx43, but a substantial increase with the double mutant cell pairing with Cx43, when compared to wildtype Cx26/Cx43 cell pair (Figure 2.2B). Calculated transjunctional conductance (G\textsubscript{j}) of heterotypic pairs Cx26/Cx43 was very low (0.27 ± 0.09 nS, n = 20), however the probability of observing coupling was evidently higher than the negative controls (50% vs 0%, n = 27). Statistically single mutants K168V and N176H failed to show an increase in G\textsubscript{j}, while the G\textsubscript{j} between cell pairs expressing double mutant K168V-N176H/Cx43 was significantly increased (p < 0.001 when comparing to wildtype Cx26/Cx43 G\textsubscript{j}) (Figure 2.3C). Due to rectifications being observed for all heterotypic pairs of Cx26 mutants/Cx43, the G\textsubscript{j}s were only calculated
when the Cx26 mutant-expressing cells were at $+V_\text{jS}$ (or the Cx43-expressing cell at $-V_\text{jS}$).
Figure 2.2 Morphological and functional status of heterotypic Cx26 mutant/Cx43 channels.

(A) Superimposed green (GFP) and red (RFP) fluorescent images (left panels) and their overlay on DIC images (right panels) to show paired N2A cells successfully expressing Cx26 variant-RFP in one cell and Cx43-GFP in the other. At the cell-cell interfaces of Cx26/Cx43 cell pairs, no co-localized GJ plaque-like structures were observed. However, in cell pairs expressing K168V/Cx43, N176H/Cx43, or K168V-N176H/Cx43, co-localized yellow GJ plaque-like structures were readily identifiable (arrows). (B, C) Dual patch clamp recording was used to measure transjunctional current (I$_j$) from heterotypic N2A cell pairs expressing RFP-tagged Cx26, K168V, N176H or K168V-N176H in one and untagged Cx43 in the other. (B) Representative traces of I$_j$ at –20 mV transjunctional voltage (V$_j$) for wildtype and mutant heterotypic Cx26/Cx43 cell pairs. K168V/Cx43 and N176H/Cx43 cell pairs do not show substantial increase in I$_j$, while K168V-N176H/Cx43 pairs showed a significantly higher I$_j$ when compared to wildtype Cx26/Cx43 heterotypic channels. (C) Bar graph illustrates the transjunctional coupling conductance (G$_j$) of heterotypic GJ channels formed in cell pairs expressing RFP-tagged Cx26, K168V, N176H or K168V-N176H in one and untagged Cx43 in the other. Cell pairs expressing K168V and N176H did not show significant increase in G$_j$ when compared to Cx26/Cx43. However, K168V-N176H cell pairs showed a very prominent increase in G$_j$ when compared to Cx26/Cx43 (p < 0.001). N2A pairs with one side expressing the empty IRES-GFP vector, while the other side expressed RFP-tagged Cx26, were used to serve as negative controls for these experiments (data not shown).
2.4.4 Cx26 K168V-N176H, but not the single mutants, formed functional heterotypic gap junction channels with Cx40

To determine whether Cx26 mutants are able to form morphological and functional heterotypic GJ channels with Cx40, another member of Group 2, we repeated the same experiments with the substitution of Cx40 instead of Cx43. To observe the co-localization and formation of putative heterotypic GJs, fluorescent images were taken of tagged heterotypic cell pairs expressing Cx40 in one and Cx26 mutants in the other. No plaque-like formations were observed at the cell-cell junction for either Cx26/Cx43 or Cx26 N176H/Cx40 pairs, while the Cx26 K168V/Cx43 and the Cx26 double mutant K168V-N176H/Cx43 pairs formed putative fluorescent GJ plaques (Figure 2.3A). Dual whole-cell patch clamp analysis was conducted to test the functionality of any heterotypic channels using the RFP-tagged mutants K168V, N176H, and K168V-N176H with untagged Cx40. Consistent with their capacity of forming morphological GJ plaques, heterotypic Cx26/Cx40 cell pairs did not display any detectible Ij during a Vj pulse (~20 mV), only 3/17 cell pairs with N176H/Cx40 showed coupling with an extremely low level of Gj. Although the K168V/Cx40 cell pairs showed morphological GJ plaques, and relatively high percentage of coupled pair (14/18), the average Gj was not statistically different from that of Cx26/Cx40. Only the K168V-N176H/Cx40 pairs showed a high coupling percentage (18/23) and substantially increased Ij during the Vj pulse (Figure 2.3B). Further Gj analysis of the heterotypic cell pairs, only K168V-N176H/Cx40 displayed a significant increase in Gj (p < 0.01 when compared to wildtype Cx26/Cx40 Gj) (Figure 2.3C). The heterotypic cell pairs with single mutants (K168V and N176H)
with Cx40 did not statistically increase the Gj, however the K168V/Cx40 pairs did display a higher percentage of cell pairs that were GJ coupled.
**Figure A**

Comparative images of Cx26/Cx40, DIC, K168V/Cx40, DIC, N176H/Cx40, K168V-N176H/Cx40.

**Figure B**

Graphical representation of voltages (-20 mV, 0 mV) and current (2 s, 0.5 nA) with corresponding channel mutations Cx26 / Cx40, N176H / Cx40, K168V / Cx40, K168V-N176H / Cx40.

**Figure C**

Bar chart showing Gj (nS) values for Cx26/Cx40, K168V/Cx40, N176H/Cx40, K168V-N176H/Cx40 with respective ratios.
Figure 2.3 Morphological and functional status of heterotypic Cx26 mutant/Cx40 channels.

(A) Superimposed green (YFP) and red (RFP) fluorescent images (left panels) and their overlay on DIC images (right panels) to show paired N2A cells successfully expressing Cx26 variant-RFP in one cell and Cx40-YFP in the other. At the cell-cell interfaces of Cx26/Cx40 or N176H/Cx40 cell pairs, no co-localized (yellow) GJ plaque-like structures were observed. However, in cell pairs expressing K168V/Cx40, or K168V-N176H/Cx40, co-localized yellow GJ plaque-like structures were readily identifiable (arrows). (B, C) Dual patch clamp recording was used to measure transjunctional current ($I_j$) from heterotypic N2A cell pairs expressing RFP-tagged Cx26, K168V, N176H or K168V-N176H in one and Cx40-IRES-GFP in the other. (B) Representative traces of $I_j$ at –20 mV transjunctional voltage ($V_j$) for wildtype and mutant heterotypic Cx26/Cx40 cell pairs. K168V/Cx40 and N176H/Cx40 cell pairs do not show substantial increase in $I_j$, while K168V-N176H/Cx40 pairs indicate a slight yet noticeable increase in $I_j$ when compared to wildtype Cx26/Cx40 heterotypic channels. (C) Bar graph illustrates the transjunctional coupling conductance ($G_j$) of heterotypic GJ channels formed in cell pairs expressing RFP-tagged Cx26, K168V, N176H or K168V-N176H in one and Cx40-IRES-GFP in the other. Cell pairs expressing K168V and N176H did not show significant increase in $G_j$ when compared to Cx26/Cx40. However, K168V-N176H cell pairs showed a prominent increase in $G_j$ when compared to Cx26/Cx43 (p < 0.01).
2.4.5 Heterotypic Cx26 K168V-N176H/Cx43 and K168V-N176H/Cx40 displayed different asymmetric \( V_j \)-gating properties.

The Cx26 K168V-N176H double mutant significantly increased the efficiency of heterotypic channel formation between Cx26 and Cx43 (or Cx40). This provided us the unique opportunity to investigate heterotypic channel properties between previously incompatible connexins. Macroscopic traces obtained from the heterotypic K168V-N176H/Cx40 and K168V-N176H/Cx43 channels displayed different signature and \( V_j \)-gating characteristics between these two heterotypic GJ channels (Figure 2.4A). The K168V-N176H/Cx40 heterotypic channels showed large extent \( V_j \)-gating with rapid gating kinetics (especially on higher \( V_j \)s) when +\( V_j \) pulses were applied on the cell express Cx26 double mutant (or –\( V_j \) on the cell express Cx40), while minimum to moderate \( V_j \)-gating with much slower gating kinetics were observed when –\( V_j \) pulses were applied on the cell express Cx26 double mutant (or +\( V_j \) on the cell express Cx40).

The amplitudes of the initial peak junctional currents (\( I_{j,ini} \)) were plotted with \( V_j \) (Fig. 2.4B) and showed a near linear I-V curve (Fig. 2.4B). The plot of \( G_{j,ini} (-)/G_{j,ini} (+) \) with \( V_j \)s for this heterotypic GJ channel also confirmed that there was no rectification of the initial \( G_j \) between the two \( V_j \) polarities.

The heterotypic K168V-N176H/Cx43 channel did not show any \( V_j \)-gating in the \( V_j \)s within ±60 mV. A low level \( V_j \)-gating with very slow \( V_j \)-gating kinetics (not even reaching an apparent steady state at the end of 7 s \( V_j \) pulses) was evident when large +\( V_j \)s (80 or 100 mV) were applied on the cells expressing the Cx26 double mutant (or –\( V_j \) was applied on cell expressing Cx43), and displayed no \( V_j \)-gating when +\( V_j \) was applied on
cell expressing Cx43 (or −Vj was applied on cell expressing the Cx26 double mutant). The amplitude of initial junctional current (Ij,ini) were plotted with Vj and showed strong inward rectification (Fig. 2.4B). The plot of Gj,ini (−)/Gj,ini (+) with Vj's for this heterotypic GJ channel further demonstrated that there was a Vj-dependent rectification of the initial Gj between the two Vj polarities. Though not every heterotypic pair of single Cx26 mutants with Cx43 (K168V/Cx43 or N175H/Cx43) showed good Gj level for Vj-gating analysis, whenever they did, both heterotypic channels showed near identical Vj-gating properties as the double mutant/Cx43 channel (Fig. 2.4A).
**Figure 2.4 Macroscopic current analysis of heterotypic K168V-N176H/Cx43 and K168V-N176H/Cx43 channels.**

(A) Dual patch clamp recording was used to obtain representative $I_j$ traces from heterotypic N2A cell pairs expressing RFP-tagged K168V, N176H or K168V-N176H in one and untagged (via IRES-GFP) Cx40 or Cx43 in the other, in response to the given $V_j$ protocol (above). (B) Initial $I_j$s from cell pairs expressing heterotypic K168V-N176H/Cx43 (open circles) and K168V-N176H/Cx40 (filled circles) were plotted against their corresponding $V_j$s to obtain the $I_j$ - $V_j$ plot. K168V-N176H/Cx43 channels indicate rectification, with lower $I_j$s recorded when $+V_j$ was applied on the cell expressing Cx43 (or $-V_j$ was applied on the cell expressing the Cx26 double mutant). (C) The ratio of initial $G_j$ ($G_{j,ini}$) values between increasing $-V_j$s and $+V_j$s (on Cx43 expressing cell) support the presence of K168V-N176H/Cx43 rectification. Cell pairs expressing K168V-N176H/Cx43 channels showed an increasing disparity between $I_j$s at increasing positive and negative $V_j$s, whereas K168V-N176H/Cx40 cell pairs stayed close to the baseline $G_{j,ini} (-)/G_{j,ini} (+)$ ratio of 1 at increasing $V_j$s.
2.4.6 Initial Gj rectification of K168V-N176H/Cx43 channels were also observed at unitary channel currents

Single channel recordings of both Cx26 mutant/Cx40 (or Cx43) heterotypic pairs were analyzed to observe if consistent channel characteristics persist at both the macroscopic and microscopic level. Cx26 K168V-N176H/Cx40 unitary channel currents ($i_{j,s}$) were readily obtainable and the $i_{j}-V_{j}$ relationship appeared to be linear, similar to those observed for the macroscopic $I_{j}-V_{j}$ relationship (Fig. 2.5B). The slope unitary conductance ($\gamma_{j}$) at both $V_{j}$ polarities were not statistically different (193.5 ± 18.7 pS for $+V_{j}$ on cell expressing Cx40, $n = 4$ and 196.9 ± 32.2 pS when $-V_{j}$ was on Cx40-expressing cell, $n = 4$). The $\gamma_{j,ini}(-)/\gamma_{j,ini}(+)$ plot also indicated no rectification, similar to its macroscopic $G_{j,ini}(-)/G_{j,ini}(+)$ counterpart (Fig. 2.5C).

Cx26 K168V-N176H/Cx43 $i_{j}$s were more difficult to obtain, due in large part to the high efficiency of forming these heterotypic channels. In the two cell pairs we were able to obtain unitary channel currents, they showed rectification in line with their macroscopic equivalent, with lower $i_{j}$s recorded when $-V_{j}$s were applied on the cell expressing the Cx26 double mutant (or $+V_{j}$s were applied on the cell expressing Cx43), and displayed comparably higher $i_{j}$s when $-V_{j}$ was applied on the cell expressing Cx43 (or $+V_{j}$ was applied on the cell expressing the Cx26 double mutant). In order to calculate slope $\gamma_{j}$ at different $V_{j}$ polarities, linear regression of $+V_{j}$ and $-V_{j}$ at 60 – 100 mV were used. While these slope $\gamma_{j}$ values showed notable difference, statistical analysis could not be conducted due to the limited number of recordings obtained (93.5 pS average for $+V_{j}$, $n = 2$ and 144.3 pS average for $-V_{j}$, $n = 2$ when on Cx43-expressing cell). While it was
difficult to acquire single channel traces for the double mutant, we were able to readily obtain $i_j$ for single mutant K168V/Cx43 channels. Similar to K168V-N176H/Cx43, rectification was also observed for K168V/Cx43 channels. The $i_j$-$V_j$ relationship supported the presence of rectification, with slope $\gamma_j$ being significantly different between $+V_j$ and $-V_j$ polarities at 60 – 100 mV linear regression ($71.7 \pm 11.5$ pS for $+V_j$ on cell expressing Cx43, $n = 4$ and $178.2 \pm 14.1$ pS when $-V_j$ was on Cx43-expressing cell, $n = 4$). We also report slope $\gamma_j$ for near 0 $V_j$ (–40 to +40 mV) due to its physiological relevance in vivo ($129.8 \pm 6.3$ pS for $V_j$ on cell expressing Cx43). The plot $\gamma_{j,ini}(-)$/$\gamma_{j,ini}(+)$ for K168V/Cx43 heterotypic channels further demonstrated that there was a $V_j$-dependent rectification of the initial $\gamma_j$ between the two $V_j$ polarities.
Figure 2.5 Single channel current analysis of heterotypic K168V-N176H/Cx40 and K168V/Cx43 channels.

(A) Dual patch clamp recording was used to obtain representative unitary channel currents (iₗ) from heterotypic N2A cell pairs expressing RFP-tagged K168V or K168V-N176H in one and IRES-eGFP Cx40 or Cx43 in the other, in response to the given Vₗ protocol (above). (B) The iₗ from cell pairs expressing heterotypic K168V/Cx43 (open circles) and K168V-N176H/Cx40 (filled circles) were plotted against their corresponding Vₗ to obtain the iₗ - Vₗ plot. Vₗ are according to the Iᵢ recording cell. K168V/Cx43 channels show rectification, with lower iₗ recorded when +Vₗ was applied on the cell expressing Cx43 (or -Vₗ was applied on the cell expressing the Cx26 mutant). (C) The ratio of unitary conductance (γᵢ) values between increasing -Vₗ and +Vₗ support the presence of K168V/Cx43 rectification. Cell pairs expressing K168V/Cx43 channels showed an increasing disparity between iₗ at increasing positive and negative Vₗ, whereas K168V-N176H/Cx40 cell pairs stayed close to the baseline γᵢ (-)/γᵢ (+) ratio of 1 at increasing Vₗ.
2.5 Discussion

The present study is based on atomic structural information of Cx26 channel and the sequence alignment analysis between docking compatible and incompatible connexins to hypothesize that the HB-forming docking residues in the Cx26 E2 domain are also important for docking incompatibility to other connexins. We designed two single mutants (K168V and N176H) and one double mutant (K168V-N76H) in Cx26 to mimic the common residues at the equivalent positions in the incompatible connexins (such as Cx40 and Cx43). Our results indicated that both single and double mutants of Cx26 increased morphological heterotypic GJs with Cx43 and Cx40. More importantly, the Cx26 double mutant established functional coupling with members of non-docking compatible connexins, both Cx43 and Cx40. To our knowledge that we are the first to convert docking incompatible connexins into compatible, indicating only few residues at the docking interface of Cx26 are important for docking preference and ability to form functional GJ channel. The specific docking compatibility of different connexins may play an important role in restricting gap junctional intercellular communication. Understanding the docking mechanisms in the compatible and incompatible connexins will help to learn their normal physiology and why these docking residues are hotspots for disease-linked mutants in many of these connexins (Bai & Wang, 2014).

2.5.1 Structural insights of docking in members of Group2 connexins

It is interesting that Cx26 K168V-N176H were able to form homotypic channels, since homology modeling predicts that homotypic K168V-N176H interactions should result in the loss of 4 of 6 HBs at the E2 docking interface (Gong et al., 2013). It was
previously reported that a loss of more than 2 HBs at the E2 interface between Cx26 and Cx32 should abolish the formation of functional channels. However, homotypic K168V-N176H channels were significantly higher in G_j when compared to the negative control (Figure 2.1C), further supporting our claim that the identity of the residues at these two positions play a critically role in determining the docking compatibility of connexins.

It is not clear what type of a non-covalent interaction(s) K168V-N176H promotes at the docking interface. One theory suggests that by changing the 167 and 175 residues on Cx32 (equivalent to positions 168 and 176 in Cx26) to be more hydrophobic, Cx32 (Group 1) can be made to heterotypically couple more like a Group 2 connexin (Harris, 2001). The author further expands on this theory by suggesting that HB interactions between K167 and N175 in Cx32 may favor their concurrence, while the more hydrophobic interactions between equivalent positions in Group 2 connexins (V and H, respectively, for Cx40) might favor their own. The results from our study happen to be in line with this theory, however we cannot be certain of the specific interactions that are at play until we acquire the atomic structure for a member of Group 2. Until such high-resolution images can be obtained, the next step would be to use homology modeling to assist in deciphering the putative interactions between heterotypic K168V-N176H/Cx43 and K168V-N176H/Cx40 channels. These interactions can then be compared to wildtype Cx26/Cx43 and Cx26/Cx40 homology models to observe potential differences.
2.5.2 Docking residues in Cx26 and their equivalent residues in other connexins are mutational hotspots for disease-linked connexin mutants

It should come as no surprise that HB forming residues responsible for docking are mutational hotspots and have been linked to a number of disease states in humans. Mutations in these HB forming (or equivalent) positions of both Group 1 and Group 2 members have been found to cause pathologies such as oculodentodigital dysplasia (ODDD), X-linked Charcot-Marie-Tooth syndrome (CMTX), and non-syndromic hearing loss (Dubourg et al., 2001; Primignani et al., 2003; Vitiello et al., 2005). An interesting study showed that a designed complementary mutant Cx26 D179N could rescue the function of CMTX linked mutant Cx32 N175D, predicted to restore a substantial number of previously lost HBs at the E2-E2 interface (Gong et al., 2013). Designed mutants have previously shown to be successful in rescuing function in other cellular channels as well (Craven & Zagotta, 2004; Haitin et al., 2013). The prospect of rescuing connexin mutants is an exciting one, considering many connexin mutations are linked to human diseases. It is currently easier to design such rescues for Group 1 connexins, due to a better grasp of their structure-function interactions (Maeda et al., 2009; Nakagawa et al., 2011). Improving our understanding of the Group 2 docking interface could allow us to more efficiently use these connexins in potential rescue studies.
2.5.3 Physiological and pathological implications of docking between Cx26/Cx43

Every cell type in the human body expresses a different set of connexins, allowing for the potential physiological interaction of many combinations of connexins. For example, Cx26 and Cx43 are co-expressed in many cell types, such as thyroid cells (Meda et al., 1993), epidermal cells (Risek et al., 1994), hypothalamic neuronal cell lines (Charles et al., 1996), and astrocytes (Nagy et al., 2001), allowing for many opportunities to communicate in a physiological setting. The functional relationship between these two connexins can have important consequences for the physiological environment at which they are expressed. The interactions of heterotypic Cx26 and Cx43 channels have previously been investigated using rodent connexins (Elfgang et al., 1995; White et al., 1995), however the electrophysiological relationship between the human counterparts to these connexins had not been elucidated. An interesting relationship was observed between several disease-linked Cx26 mutants and Cx43. A number of E1 mutants in Cx26 were found to exert a trans-dominant negative inhibition on Cx43, while other Cx26 mutants promoted the formation of hyperactive heteromeric hemichannels with Cx43 (Garcia et al., 2015; Rouan et al., 2001). These Cx26 mutants were associated with palmaplantar keratoderma and keratitis-ichthyosis-deafness syndrome, respectively.

The presence of compatible/incompatible connexins raises the question of why there are multiple groups of connexins. Cells from the same tissue usually express the same combination of connexins, and predominantly form channels that are homomeric and homotypic. While less common, formations of heterotypic GJ channels have been
found to occur in a number of native cells, namely in the liver (Cx26/Cx32) (Sosinsky, 1995), heart (Cx45/Cx43 and Cx45/Cx40) (Bukauskas et al., 2006), and during astrocyte-oligodendrocyte interactions (Cx30/Cx32, Cx43/Cx45, and Cx45/Cx30) (E. Dahl et al., 1996; Elfgang et al., 1995; Harris, 2001; Manthey et al., 2001). While heterotypic interactions exist, connexins are often limited to forming functional channels with members of their own docking compatible group. A potential justification for this functional barrier might be to compartmentalize and isolate certain cell types from others within a tissue or organ, which might be necessary for certain physiological processes. Another possibility could be that the incompatibility observed between some heterotypic channels might not be as absolute as we might believe. We recorded non-significant conductance for wildtype Cx26/Cx43 channels, however these channels exhibited notable coupling (50%) when compared to the negative control and Cx26/Cx40 channels (both of which were 0%). This level of coupling made us wonder whether Cx26/Cx43 channels may play a subtle yet important role in cell-to-cell communication and synchronization in native tissues. Connexins are believed to have a high level of redundancy, as was seen when Cx43 was knocked-down in a mouse model (van Rijen et al., 2004). The level of conductance in the ventricle was not altered when the expression of Cx43 was dropped to 50%, and only decreased by 15% and 25% at knockdowns of 70% and 95%, respectively. Such a high level of redundancy could mean that even relatively low amounts of coupling could play a physiological role in tissues. It would be interesting to see the effects that a gain of function between Cx26 and Cx43 would have in vivo.
2.5.4 Experimentally determined high-resolution crystal structure of Cx26 is an excellent template to understand GJ function

The high-resolution structure of Cx26 has previously been used as a template for the development of homology models for other connexins. For example, Cx26 was previously used as a template to model Cx32 due to the high sequence identity between the two connexins (69% in entire sequence alignment) (Nakagawa et al., 2011). Working with the established structure of Cx26 along with the putative model of Cx32, (Gong et al., 2013) altered a number of critical residues responsible for HB formation between apposing hemichannels in order to gain further insight to the role and influence of HBs in docking. Another study used a Cx50 homology model to investigate the influence of E1 mutants on unitary conductance and voltage-dependent gating (Tong et al., 2014).

Homology modeling has also been used for Group 2 connexins, Cx40 and Cx43, during a study aimed at determining whether these two connexins form functional heterotypic GJ channels, and to identify potential residues responsible for their docking (Jassim et al., submitted). In short, homology models stand as a reliable tool in determining putative structures for connexins for which we do not yet have high-resolution structure.

It is important to note that our study is the first to alter HB forming residues of the E2 domain with the specific goal of investigating Group specific compatibility. Currently, we have limited structural information for Group 2 connexins, making it difficult to predict interactions at the molecular level. This is especially the case regarding our understanding of head-to-head docking when concerned with hemichannels composed of Group 2 connexins. Our study helps to close this gap of knowledge.
other members of their family, in compositions that can be heteromeric, heterotypic, or both. Answering the question of what determines heterotypic connexin compatibility is important in expanding our fundamental understanding of these channel proteins. The results we have obtained can help guide subsequent research on establishing docking interactions of Group 2 connexins. Furthermore, understanding these fundamental interactions between connexins can allow for easier downstream manipulations in enhancing/decreasing heterotypic GJ function by gene therapy approaches.

2.5.5 Conclusion

It has been long established that the E2 domain is important for non-covelant interactions between two docked hemichannels. Our study provides experimental evidence for the important role that two positions on the E2 domain play in heterotypic docking between connexins from different Groups. Cx26 (Group 1) does not naturally form functional heterotypic gap junction channels with Cx43 or Cx40 (Group 2). Mutating two critical residues on the E2 domain (K168 and N176) of Cx26, to match the residues expressed in the opposite Group, resulted in substantial increase in heterotypic function when compared to their respective wildtype counterparts. These two positions could be responsible for creating a functional barrier between connexins of different Groups. Further studies must be conducted to see if other members of Group 1 and Group 2 behave similarly when residues at equivalent positions are altered in the same manner. Our results suggest the presence of two key residues in the process of heterotypic functional selectivity for Cx26.
2.6 References


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Chapter 3 - Discussion

3.1 Overall Study

This study investigated the electrophysiological properties of heterotypic docking between human Cx26 with Cx43 and Cx40, and the effects of altering positions K168 and N176 of Cx26 in heterotypic compatibility with Cx43 and Cx40. Co-expression of Cx26 and Cx43 is seen in a number of different tissues and organs in humans (Brehm et al., 2002; Monaghan et al., 1996; Salomon et al., 1994). Initial studies looking at Cx26/Cx43 heterotypic gap junction (GJ) channels used rodent connexins and dye coupling to identify the presence of any interactions (Tomasetto et al., 1993; White et al., 1995). However, these studies lacked sensitive electrophysiological analysis and did not investigate the human counterparts to these connexins. Here we describe the electrophysiological function analysis of Cx26/Cx43 and Cx26/Cx40 heterotypic channels, using dual whole-cell patch clamp technique. Cx43 and Cx40 belong to different compatibility Groups than Cx26, however the incompatibility between these Groups have yet to be explained at the molecular level (Bai & Wang, 2014). We were able to establish that the amino acid identity at two critical positions (K168 and N176) on the Cx26 E2 domain were responsible for heterotypic compatibility.
3.2 Human Cx26/Cx43 and Cx26/Cx40 channels do not show high conductance

Transjuncional conductance (Gj) analysis for wildtype heterotypic Cx26/Cx43 and Cx26/Cx40 GJ channels did not indicate significant conductance when compared with controls (Chapter 2, Figure 2.2C and 2.3C). Interestingly, Cx26/Cx43 channels did show high amount of coupling (50%) compared to Cx26/Cx40 channels (0%). These Cx26/Cx43 channels also showed rectification similar to the K168V/Cx43 channels that were reported (Chapter 2, Figure 2.4A). Our observations partly agree with previous studies, which indicated that rodent Cx26 form non-functional heterotypic gap junction (GJ) channels with Cx43 and Cx40 (Elfgang et al., 1995; White et al., 1995). While the channel conductance in our study is also very low, the high level of heterotypic coupling between Cx26 and Cx43 cannot be overlooked. However, it is difficult to ascertain the influence such low conductance/ high coupling channels might have in a physiological setting. It is worthwhile to point to the important gap that our study fills – which is the combined use of both human connexins and patch clamp analysis for determining heterotypic Cx26/Cx43 and Cx26/Cx40 GJ function.

3.3 K168 and N176 influences heterotypic docking in Cx26

In this paper, we report that the Cx26 double mutant, K168V-N176H, altered Cx26 heterotypic compatibility by allowing functional docking with Cx43 and Cx40. Single mutants K168V and N176H greatly lowered the homotypic function of Cx26, yet did not result in a significant enough increase in heterotypic compatibility when allowed to dock with Cx43 or Cx40. However, the presence of K168V was enough to observe
morphological plaque-like structures when docked with either Cx43 or Cx40. N176H, on the other hand, only showed putative plaque formation with Cx43, but not Cx40. Previous analysis on homotypic Cx26 K168V and Cx32 N175H, which is equivalent to Cx26 N176H, was conducted to investigate the influence of docking hydrogen bonds (HBs) in functional GJ formation (Gong et al., 2013). Our G_j recording for homotypic Cx26 N176H was similar to published Cx32 N175H. However, Gong et al. (2013) observed higher G_j8 for their Cx26 K168V recordings when compared to ours. This discrepancy could be due to differences in the incubation times, as Gong et al. (2013) used overnight incubation whereas we incubated the cells for 1-2 hours before applying patch clamp recording. To be certain that time was not a limiting factor, we did use longer incubation times (> 6-8 hours) for heterotypic combinations reporting low G_j. However, we did not observe a notable difference after longer incubation times (data not shown).

Docking of double mutant K168V-N176H with Cx43 and Cx40 resulted in high G_j heterotypic channels when compared to wildtype Cx26/Cx43 and Cx26/Cx40 (Chapter 2, Figure 2.3C and 2.4C). Homotypic K168V-N176H additionally formed functional, yet lower G_j, channels when allowed to dock. Homology/functional studies suggest that K168V-N176H should lose 4/6 docking HBs, which would result in the abolishment of docking and subsequent functional channel formation (Gong et al., 2013). However, this was not the case as the G_j for homotypic K168V-N176H was halfway between the negative control and homotypic Cx26, and significantly different from both (p < 0.05 in both cases). In comparison, N176H is also predicted to result in the loss of 4/6 docking HBs, yet has considerably lower G_j when compared to N176H-K168V. A possible
explanation could be that the presence of the K168V and N176H mutants introduce non-cova
celant interactions that compensate for the loss of docking HBs. Homology modeling can assist in identifying the presence of such novel interactions. Since our hypothesis assumes Cx40 and Cx43 (Group 2) have similar structure and use similar residues in docking as Cx26 (Group 1), we can claim that the novel interactions we identify are unique to the Group 2 docking mechanism. It is important to note that such assumptions are limited to the accuracy of the homology model we are working with, however previous studies have shown Cx26 to be a reliable structural template for other connexins (Gong et al., 2013; Nakagawa et al., 2011; Tong et al., 2014).

Macroscopic traces were observed when K168V-N176H was allowed to dock with Cx43 and Cx40. When paired with Cx43, K168V-N176H formed channels with distinct rectification. Rectification is an electrical property observed when there is asymmetrical transmission, which results in differential resistance to current flow in one direction versus the other. Such asymmetrical transmission can be observed in the case of heterotypic GJs with two distinct hemichannels, such as in the presence of Cx34.7/Cx35 and Cx26/Cx32 heterotypic channels (Rash et al., 2013; Rubin et al., 1992). Rectification observed for K168V-N176H/Cx43 channels could be caused by the opposite gating polarity of Cx26 and Cx43. GJ channels composed of hemichannels with opposite gating polarity can exhibit rectification, because while one $V_j$ polarity opens both hemichannels, the opposite polarity closes them (Verselis et al., 1994). For the case of K168V-N176H/Cx40, no rectification was observed, however the channel showed asymmetric gating. A number of factors could promote asymmetric gating in heterotypic channels,
such as gating polarity, differences in hemiclannel unitary conductance ($\gamma_j$) and differences in intrinsic sensitivity to $V_j$ (Bukauskas et al., 1995; Rackauskas et al., 2007).

### 3.4 Physiological and pathological role of Cx26 and Cx43 interaction

It is important to establish an accurate understanding of connexin compatibility. In most cell types, different combinations of connexin isoforms are co-expressed. Cx26 and Cx43 are found together in a number of different tissues and organs throughout the human body, such as in the skin (Salomon et al., 1994), testes (Brehm et al., 2002), and mammary epithelium (Monaghan et al., 1996). While they are co-localized to a great extent, wildtype Cx26 and Cx43 have yet to be shown to assemble into heteromeric hemiclannels (Beyer et al., 2001; Gemel et al., 2004; Jara et al., 2012). It is not certain why Cx26 and Cx43 cannot form heteromeric hemiclannels, however studies have suggested that certain differences in structure or post-translational trafficking and modification might play a role. There are two important differences between Cx26 and Cx43 worth noting; Cx26 has a shorter C-terminal tail and is neither phosphorylated nor glycosylated, which suggests that Cx26 might be trafficked to the membrane through alternative pathways (Delmar et al., 2004; Maeda et al., 2009). However, cell culture studies have reported conflicting results regarding Cx26 and Cx43 trafficking (Gemel et al., 2004; Martin et al., 2001; Thomas et al., 2001). One explanation is that these studies did not use a unifying cell culture model, and different cell models might be trafficking these proteins via different pathways. It is also possible that other factors beyond our current knowledge influence the trafficking control of these connexins, which is very likely considering connexins are involved in the formation of a myriad of multiprotein complexes (Dbouk et al., 2009; Laird, 2010).
Interestingly, co-expression of Cx26 and Cx43 in the same cell was found to reduce total transjunctival conductance \( (G_j) \) to 10% of what is observed when only a single connexin is expressed (Cx26 alone, or Cx43 alone) (Gemel et al., 2004). In contrast, co-injection of identical amounts of Cx26 and Cx43 mRNA into *Xenopus* oocytes resulted in a significant increase in intercellular communication, suggestive of an additive effect (Rouan et al., 2001). The contradicting results observed between these two studies could be due to a number of factors, such as the use of different cell models, expression protocols (transfection or injection), and the use of rodent versus human connexins. One of the most interesting findings from Rouan et al. (2001) was the drop in GJ conductance (down to 5-20% compared to wildtype co-expression) that was observed when palmoplantar keratoderma/ hearing impairment (PPK/HI) associated Cx26 mutants were co-expressed with Cx43. These PPK/HI mutants not only impaired function of wildtype Cx26 channels, but also exerted a trans-dominant negative effect on co-expressed Cx43. Such an inhibitory interaction would explain why certain Cx26 mutants cause only HI, while others also show symptoms of skin diseases, such as PPK and Keratitis-Ichthyosis-Deafness (KID) (Martinez et al., 2009). However, the actual interactions between these Cx26 PPK/HI mutants and Cx43 are unclear. One possibility is that Cx26 mutants physically hinder the formation of Cx43 channels and limit the maximum size of GJ plaques (Bukauskas et al., 2000). Another explanation could be that PPK/HI mutants sequester Cx43 by promoting abnormal formation of heteromeric hemichannels, while impairing GJ formation and function. The plausibility of this theory was shown in a recent study conducted by Garcia et al. (2015), where they discovered that a number of autosomal dominant Cx26 mutants associated with the KID syndrome formed hyperactive heteromeric hemichannels with wildtype Cx43, while showing
reduced or eliminated GJ channel formation. These hyperactive hemichannels were found to cause intracellular Ca$^{2+}$ overload and ATP release when compared to cells co-expressing wildtype counterparts. In the KID syndrome, the most extensive damage is usually observed in the epidermis, where both Cx26 and Cx43 play a crucial role in keratinocyte homeostasis and maintenance (Risek et al., 1992). ATP signaling is important in keratinocyte differentiation and proliferation (Denda et al., 2002), which explains how hemichannel hyperactivity and subsequent ATP release could cause the pathological phenotype. These types of Cx26 and Cx43 interactions could be the root cause of a number of other skin diseases, making it necessary to further investigate the interaction between these connexins.

### 3.5 Potential physiological role of connexin incompatibility and compartmentalization

Gap junctional intercellular communication (GJIC) is critical in maintaining tissue and organ specific homeostasis, loss of which is a hallmark of transformation and cancer (Sułkowski et al., 1999). While the importance of GJIC is undeniable, a lack of such communication may also play a significant role in shaping and maintaining normal physiological function. Connexins from different compatibility groups have been found to co-exist in the same tissue and organs without functionally interacting (Gemel et al., 2004; Smith et al., 2012). This raises the question of why these incompatibilities originated, and to what extent different connexin isoforms limit or support physiological specialization of different cell types.
Connexin expression in the mammary glands has been an interesting area of study. In the rodent mammary gland, connexin distribution follows an asymmetric pattern where Cx26, Cx32 and Cx30 are expressed in the luminal epithelium while only Cx43 is expressed in the myoepithelium (Locke et al., 2004; Talhouk et al., 2005). This provides us with the unique opportunity to investigate physiologic functional interactions between connexins belonging to different compatibility groups, since Cx26, Cx32 and Cx30 are members of Group 1 and Cx43 is a member of Group 2. In line with previously mentioned cell culture studies, it was shown that ectopic expression of Cx26 in the myoepithelium altered the expression of endogenous Cx43 and led to impairment in milk delivery (Mroue et al., 2015). Loss of Cx43 was also shown to adversely affect oxytocin-mediated myoepithelial contraction in the mammary gland. In fact, a similar observation regarding the role of Cx43 in the loss of smooth muscle contractility was previously made in other oxytocin sensitive tissues, such as the uterine myometrium (Doring et al., 2006). Currently, only Cx26 and Cx43 have been identified in the human breast, however these findings are subject to certain limitations (McLachlan et al., 2007). Human mammary gland tissues are hard to come by, and so far those that have been investigated have belonged to non-pregnant female adults. The lack of mammary gland tissues from different stages of development and pregnancy make it difficult to conclude that Cx26 and Cx43 are the only connexins that are found in the human breast. These findings do indicate that a certain degree of compartmentalization due to connexin incompatibility may play a role in maintaining normal physiological functions.

Of known connexins, Cx43 is the most extensively expressed throughout the body in many tissues and organs. Studies have shown that Cx43 knockouts (Cx43\(^{-/-}\)) result in
postnatal lethality due to obstruction of ventricular outflow (Reaume et al., 1995). Cx43−/− was also shown to result in other developmental abnormalities, such as impairment of spermatogenesis in the testes (Roscoe et al., 2001). The postnatal lethality of Cx43−/− could be partially rescued by knock-in of Cx32 or Cx40, however such knock-ins still showed functional and morphological abnormalities (Plum et al., 2000). Winterhager et al. (2007) conducted a similar knock-in study using Cx26 to replace the coding region of Cx43 in mice. They successfully generated mice that either solely expressed (homozygous, Cx4326/26) or co-expressed (heterozygous, Cx4326/43) Cx26 in cells that endogenously express Cx43. Dominant impairment of mammary function was observed in Cx4326/43 mice, supporting previous findings that reported negative interaction between co-expressed Cx26 and Cx43. It was previously shown that Cx43 hemizygous (Cx43+/−) mice do not have any lactation problems, indicating that it was the substitution of Cx26 that compromised mammary gland function, not the decreased level of Cx43 (Plum et al., 2000). Furthermore, examined Cx4326/26 mice were found to be infertile, and histological analysis indicated that the differentiated stages of spermatogenesis were absent. Even with the observed abnormalities, the study claims that Cx26 can partially replace Cx43 in function, simply because heterozygous Cx4326/43 mice were viable, and were also able to produce viable, Cx4326/26 progeny when crossed with one another. Even though Cx4326/26 mice had improved postnatal survival compared to Cx43−/−, homozygous mice had slower ventricular conduction in the heart and showed a notable disadvantage in survival when compared to wildtype and Cx4326/43 mice, suggesting long-term impairment of myocardium function (Rouan et al., 2001). A previous knock-in study using Cx32 to replace Cx43 demonstrated impairment of mammary gland function, similar to the Cx26 knock-in study. However, the cause for the similar impairment was different in both
knock-ins. Specifically, Cx32 knock-in disturbed milk ejection, whereas Cx26 knock-in caused reduced glandular development (Plum et al., 2000). In conjunction with previous knock in studies, these results show that different connexins fulfill different roles in different cell types, yet are able to partially replace one another even when expressed in cell types they are not native to.

3.6 Limitations and future directions

This study provides novel insight into the unique role that K168 and N176 play in terms of the heterotypic docking compatibility of Cx26. However, there are limitations that need to be carefully considered when interpreting our results. The lack of high-resolution atomic structure for Cx40 and Cx43 makes it difficult to correlate our functional observations to interactions at the docking interface. At 3.5 Å, Cx26 is currently the only channel structure we have for members of the GJ family. Many important features such as $V_J$ and pH sensitivity, high conservation of both extracellular domains, and the ability of NT to determine $V_J$-gating properties are shared among GJ channels. This makes it plausible to use the structure of Cx26 as a template for homology modeling of other connexin family members. However, even with careful calculations during the generation of homology models for other connexins, it cannot be ruled out that there might be subtle differences that can influence function related interactions. These limitations would make it difficult to say with certainty what types of interactions exist in the heterotypic docking interface of wildtype and mutant Cx26 with Cx43 or Cx40.

Our current study has identified positions K168 and N176 to be critical in determining heterotypic compatibility of Cx26. However, these findings alone cannot
conclude that the equivalent positions found in other connexins play a similar role. Other Group 1 members (Cx30, Cx32, Cx46 and Cx50) are believed to share similar structure and interaction profiles as Cx26, making it likely that they also use K168 and N176 equivalent positions in heterotypic compatibility. However, the same cannot be extrapolated for members of Group 2. Our future plans involve reversing our current heterotypic protocol by altering equivalent positions in Cx43 or Cx40 to see the effect on functional heterotypic GJs formation with wildtype Cx26. This would confirm that Cx40 and Cx43 use similar residues as Cx26 when determining heterotypic compatibility. Furthermore, altering equivalent docking-HB forming residues in other Group 1 members to observe heterotypic compatibility with connexins belonging to Group 2 would further validate our hypothesis. An example for this would be altering Cx50 (Group 1 member) at the equivalent two positions we did in Cx26 (K168V and N176H), and observing to see if the Cx50 mutants dock to form functional channels with Cx37 (Group 2 member).

3.7 Summary

Here we have identified two positions, K168 and N176, in the E2 domain of Cx26 that are responsible for heterotypic compatibility. Altering these two positions together allowed for the functional heterotypic docking of Cx26 with Cx43 or Cx40, which was not previously possible. K168 and N176 are two of the four residues responsible for the formation of docking HBs in Cx26 channels. Docking HB-forming residues are critical for the formation of functional GJ channels, and have been identified as mutational hotspots across many connexin isoforms. Our findings suggest that K168 and N176 equivalent positions in Group 2 connexins are also important in docking; however their role in Group 2 specific docking mechanism has yet to be determined. While homology
models could provide valuable insight, without the support of Cx43 and Cx40 structure, it is difficult to reveal the docking mechanism in Group 2 connexins. Further studies examining the alteration of equivalent residues in different heterotypic, cross-group connexin pairs can support our claim that these two positions in the E2 domain of connexins are responsible for group-specific heterotypic compatibility.
3.8 References


dysfunctional reproductive organs and slowed ventricular conduction in the heart. 
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