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Engineered connexin40 variants increased docking and function of heterotypic connexin40/connexin43 gap junction channels

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Abstract

Gap junction (GJ) channels provide low resistance passage for rapid action potential propagation in the heart. Both connexin40 (Cx40) and Cx43 are abundantly expressed in and frequently co-localized between atrial myocytes, possibly forming heterotypic GJ channels. However, conflicting results have been obtained on the functional status of heterotypic Cx40/Cx43 GJs. Here we provide experimental evidence that the docking and formation of heterotypic Cx40/Cx43 GJs can be substantially increased by properly designed Cx40 variants where the extracellular domains (E1 and E2) have been modified. Specifically, Cx40 D55N and P193Q; substantially increased the probability to form GJ plaque-like structures at the cell-cell interfaces with Cx43. More importantly the coupling conductance (G_j) of D55N/Cx43 and P193Q/Cx43 GJ channels are significantly increased from the G_j of Cx40/Cx43. Our homology models indicate the electrostatic interactions and surface structures at the docking interface are key factors preventing Cx40 from docking to Cx43. Improving heterotypic G_j of these atrial connexins may be potentially useful in improving the coupling and synchronization of atrial myocardium.

Keywords: gap junction channel, heterotypic docking, connexin40, connexin43

Abbreviations: Cx40, connexin40; Cx43, connexin43; E1, the first extracellular domain; E2, the second extracellular domain; GJ, gap junction; $G_{j,ss}$, normalized steady-state junctional conductance; I_j, macroscopic junctional current; V_j, transjunctional voltage;

Dedication

With your affection, love, encouragement, and prayers I was able to finish my MSc project and my thesis. I dedicate this work to: my mother and my father.

Co-Authorship Statement

All of the work presented in this study was achieved by Arjewan Jassim except with some help of offering extra recordings on D55N/Cx43 and Cx40-untagged which was done by Willy Ye. The morphological data using HeLa cells was obtained and modified by Honghong Chen .

Acknowledgments

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List of Abbreviations

$[H^+]_i$ intracellular concentration of protons

ADP   adenosine diphosphate

ATP   adenosine triphosphate

AV   atrioventricular node

BBB   blood brain barrier

Ca^{2+} calcium ions

cAMP   cyclic adenosine monophosphate

CL   cytoplasmic loop

CMTX   charcot-marie-tooth syndrome

CT   carboxyl-terminus

Cx   connexin

Da   dalton

E1   extracellular loop 1

E2   extracellular loop 2

ECF   extracellular fluid

ER   endoplasmic reticulum

G_j junctional conductance

GJ   gap junctions

GJIC   gap junctional intercellular communication

HSP   hereditary Spastic Paraplegia

ICF   intracellular fluid

I_j	macroscopic junctional current
i_j	junctional current of single channel
$InsP_3$	inositol 1,4,5-trisphosphate
ms	millisecond
MW	molecular weight
N2A	mouse neuroblastoma cells
nS	nanoSiemens
NT	amino-terminus
ODDD	oculodentodigital dysplasia
SA	sinoatrial node
SEM	standard error of the mean
TM	transmembrane domain
V_j	transjunctional voltage
V_m	transmembrane voltage

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Chapter 1: Introduction

1.1 Gap junction (GJ)

Gap junctions (GJs) are clusters of intercellular channels expressed by many tissues. The GJ channels permit gap junctional intercellular communication (GJIC), which exists in almost every mammalian cell type [1]. GJs form clusters of a few to hundreds of tightly packed intercellular channels. A single GJ channel is composed of two hemichannels of adjacent cells docked together to form a direct intercellular passage. Each hemichannel (also known as a connexon) spans a single plasma membrane and consists of a hexamer of protein subunits called connexins (Cx) **(Figure 1.1)**. In general, all GJs are permeable to molecules smaller than 1000 Da [2]. GJs play important roles in diverse biological processes including synchronization of cardiac and smooth muscle during contractions [3] and immune responses [4].

Gap junction channels can be regulated through multiple mechanisms. They are voltage dependent channels with different connexins exhibiting voltage gating properties [5]. GJ channels can be closed or partially closed by the voltage difference between either the two connected pools of cytoplasm or the interior of the cell and its exterior. The difference in voltage between the two cytosols is termed transjunctional voltage (V_j) and the difference in voltage of the interior of the cell and its exterior environment is termed transmembrane voltage (V_m). The V_j can be established using dual whole cell voltage clamp technique and the transjunctional current (I_j) can be recorded. The junctional conductance, G_j, can be calculated. In addition to transjunctional voltage, intracellular Ca^{2+} concentration has been implicated as another important factor to gate GJs. An increase in the intracellular concentration of Ca^{2+} reduces several gap junction functions, possibly due to activation of calmodulin acting

as a gating molecule [6]. It has been shown that GJ channels can be closed by an increase in the intracellular concentration of H^+ [7]. The most thoroughly characterized type of posttranslational regulation of connexins is phosphorylation. Inhibition of phosphoylation of Cx43 (the dominant connexin in the heart) results in redistribution of Cx43. For example, during ischemia of the heart, the phosphorylation is decreased and the localization of Cx43 at the intercalated discs is reduced and at the same time its lateral distribution in the cardiomyocytes is increased [8].
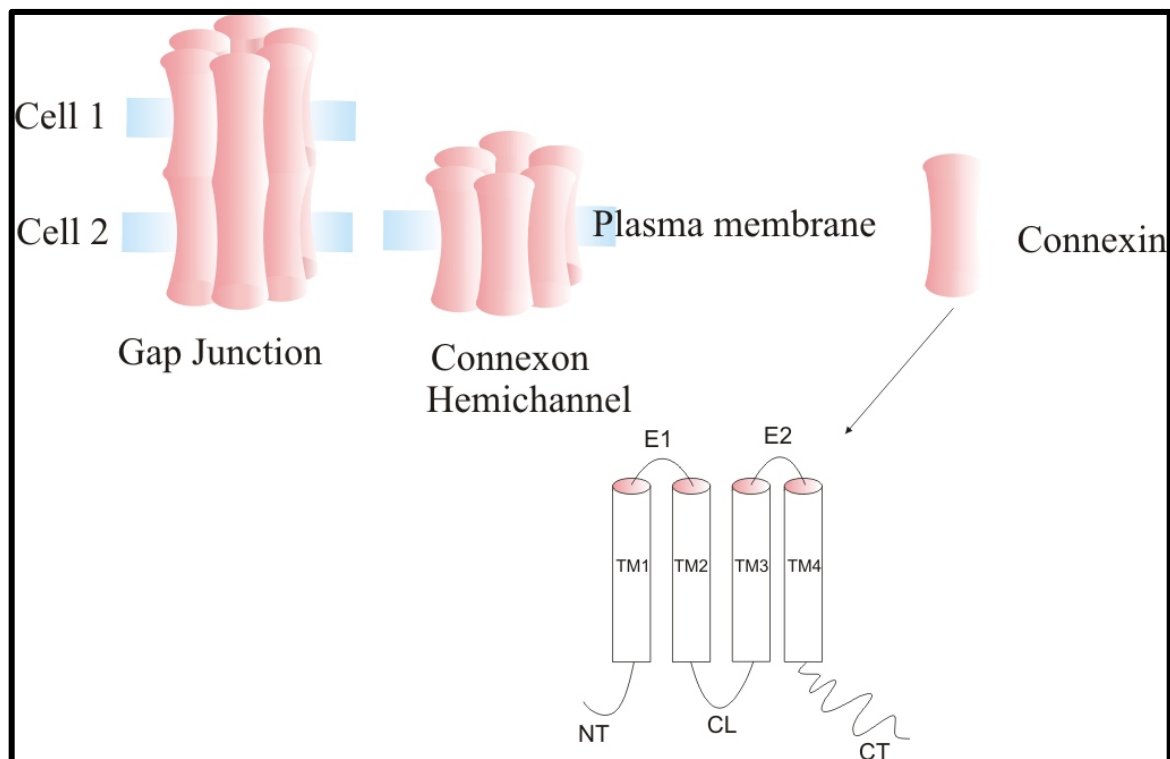
Fig. 1.1. Diagram of gap junction and a model showing the topology of connexin

A gap junction is formed by the association of two hemichannels. Each hemichannel is composed of six protein isoforms called connexins. All connexins share same structural topology, with two extracellular loops (termed E1 and E2), four transmembrane domains (TM1-TM4), a cytoplasmic loop (CL), and intracellular C-terminus (CT) and an intracellular N-terminus (NT).

1.2 Connexins

The molecular component of the vertebrate GJ channels is the connexin (Cx). A hemichannel is formed by the oligomerization of six protein subunit isoforms of connexins. To date, there are 20 connexin genes in the mouse and 21 connexin genes in the human genome. Most connexins share similar structural transmembrane topology consisting of four alpha-helical transmembrane domains (TM1-TM4), two extracellular loops (E1 and E2), a cytoplasmic loop (CL), which connects TM2 and TM3, a cytoplasmic amino-terminus (NT) and a cytoplasmic carboxyl-terminus (CT) **(Figure 1.1).** Connexins differ mainly in their length and amino acid sequence, but the transmembrane domains, amino-terminus, and the two extracellular loops show highly conserved sequences. The amino-terminus is short and it plays a role in Vj-gating, influences molecular permeability, and affects single channel conductance [9]. It has been shown that when the NT of Cx45.6, which is orthologous to Cx50, was replaced with the NT of Cx43, the Vj-gating and unitary conductance were completely altered [10]. The extracellular loops (E1-E2) are vital for the docking of hemichannels and the formation of a functional gap junction channel [11]. Different connexins exhibit distinct spatial and temporal patterns of expression. That may relate to the distinct functions of GJ channels that are composed of different connexins. GJs of different connexins exhibit a wide range permeability, unitary conductance, and Vj-gating properties. Ongoing research using sequencing analysis, structural models, and mutagenesis, in combination with functional approaches, will reveal more details regarding the structure-function relationship in GJ channels.
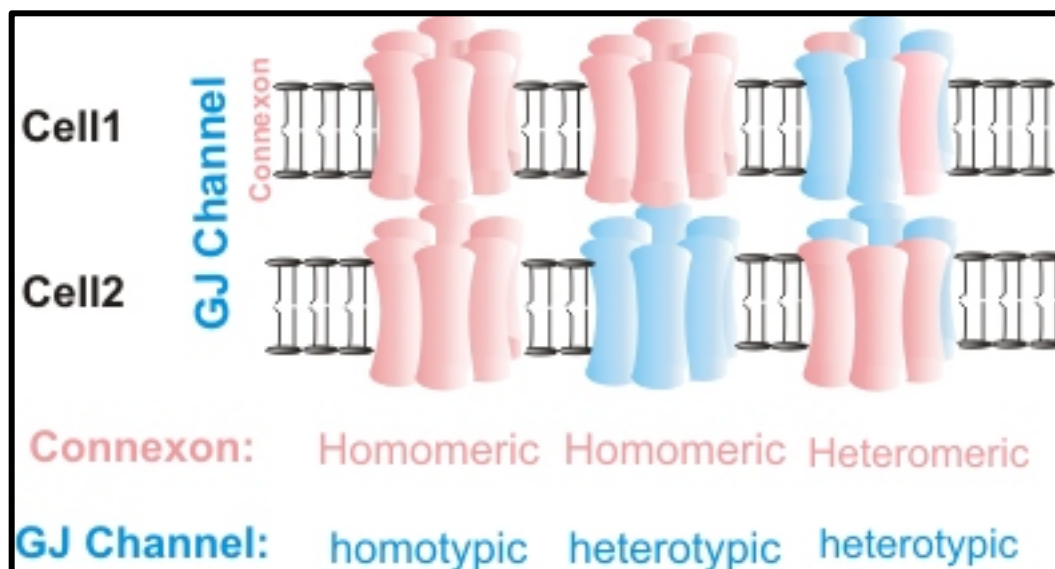
Fig. 1.2. Model of gap junction structure

Due to the different arrangements of the connexins composing the GJ channels, there are multiple forms of GJs. The diagram shows three different combinations; identical homomeric hemichannels dock together to form a homomeric homotypic GJ (first configuration); docking of two different homomeric hemichannels to form a homomeric heterotypic GJ (second configuration); hemichannel composed of non-identical connexins docking with another hemichannel formed by different ratio of connexins to make a heteromeric heterotypic GJ channel (third configuration).

1.2.1 Connexin isoforms

The predicted molecular mass of connexin is what in most cases determines their widely used nomenclature. For example, the molecular weight (MW) of Cx43 is 43kDa [12]. Connexins are classified into five families (α, β, γ, δ and ε) based on the homology sequence, and divergence in the length of the cytoplasmic loop (CL) and carboxyl-terminus (CT) [5]. Numerous cells and tissue types express connexin proteins. The co-expression of multiple connexins can generate different forms of hemichannels. The hemichannels could potentially be heteromeric formed by different connexins, or homomeric formed by same connexins **(Figure 1.2)**. Upon docking of two hemichannels, GJ channel could be homotypic or heterotypic. The homotypic GJ is formed by two identical hemichannels. The heterotypic GJ is formed by two non-identical hemichannels. Because single cell can express at least two types of connexins, heteromeric or heterotypic GJ channels can be formed. These heteromeric or heterotypic GJ channels have distinct functional properties in order to meet the physiological necessities of the organ. Many studies on heterotypic compatibility have revealed the docking selectivity among connexins. **Table 1.1** shows ten connexins that have been extensively studied in vitro for their docking compatibility in forming functional GJs. They have been divided into: Group1 and Group2. In general, intra-group connexins are compatible in docking and they can form functional GJ channels. However, in most cases, inter-group connexins are incompatible and cannot form functional GJ channels. On the other hand, some connexins belong to the same group and hold a negative/positive (− / +) sign for their compatibility (e.g. Cx40 and Cx43). The concept of the formation of functional heterotypic Cx40/Cx43 GJ was supported

by several studies [13, 14]. In contrast, other studies that published electrophysiology data on heterotypic Cx40/Cx43 GJ channels concluded that these GJs are non-functional in both *Xenopus* oocytes and in mammalian cells [11, 15]. At the moment, the ability or inability of Cx40 and Cx43 to interact and form functional GJ channel is still unclear. The main aim of this project is to resolve this controversial issue and to try to genetically engineer some Cx40 variants to improve Cx40 ability to dock with Cx43 to form functional heterotypic GJ channels.

1.2.2 Extracellular loops (E1 and E2)

Most homotypic GJ channels are functional, but not all heterotypic GJ channels can be formed and show functional communications. In another word, formation of functional heterotypic GJs is selective and depends on the component connexins composition and their compatibility. The question is, "what determines the selectivity between connexins in forming functional heterotypic GJs channels?" In early studies using chimeras' connexins swapping of extracellular loops, it was found that E2 is the primary structural element responsible for the docking selectivity and specificity [16, 17]. The E1 and E2 contain three cysteine residues in each domain, which were proposed to play a partial role in the docking selectivity, with equal spaces in between in most connexins. However, it was found that the disulfide bonds of these residues are not made for end-to-end docking hemichannels of cardiac GJs or between the connexins of the hemichannels [18, 19]. Hemichannels of GJ channels interact in interdigiting manner using non-covalent interactions to stabilizing the docked hemichannels. Specifically, hydrogen bonds (HBs) between docked E1 and E2 have been shown to exist in Cx26 channel and also in heterotypic Cx26/Cx32 GJ docking

interfaces. On average, there are 60 HBs in total between the docked two hemichannels, 24 HBs between extracellular loops (E1), and 36 HBs between the E2s [20]. When mutating specific amino acid residues located on E2, HBs number have been reduced and that affects the formation of homo- and hetero-typic GJs in Cx32 and Cx26 [20] [21]. Sequence alignment comparison of Group1 and Group2 connexins of the E1 and E2 showed that E1s are highly conversed and no clear pattern difference between these two groups of connexins. However, there are variations at E2 HB-forming residues, which might account for the connexins docking specificity in forming heterotypic GJ channels among different connexins groups. Because of the reasons mentioned above about selectivity among connexins in forming functional GJs and the participation of extracellular domains in the docking process, this project is mainly designed to generate certain mutations (variants) located on the extracellular loops (E1 and E2) aimed at improving the incompatible connexins to form operative GJs.

1.2.3 Connexin biosynthesis

Connexin protein synthesis follows the general classical secretory pathway. The mRNA of connexins in the cytosol are translated at the ribosomes of the rough endoplasmic reticulum (ER), where folding and threading into the ER occurs co-translationally [22]. Secretory vesicles bud off from the ER membrane and travel to the Cis-Golgi apparatus face. However, connexins must oligomerize before they can function. Oligomerization of the six protein subunits of connexins occur either before leaving ER or in the Golgi apparatus, depending on the connexin isoform [23]. Cx43, for example, oligomerizes after exiting the ER and in the Golgi network [24]. The

vesicles containing the hemichannels moving via microtubules will integrate with the plasma membrane presenting the hemichannels (connexons) at the extracellular surface [25, 26]. Once hemichannels are at the plasma membrane, their half-life before internalization and degradation is short and ranges between 1-5 hours. The new synthesized cardiac connexin, Cx43, will disappear within half-life at approximately 2 hours [27]. This quick appearance and disappearance allow the static cells such as the working cardiomyocytes to rapidly regulate the content of their GJs [26]. In specific situations such as ischemia and hypertrophy, the turnover of Cx43 by the ventricular myocytes would be severe and might cause alteration in propagation of electrical pulses [28]. Generally, GJs are dynamic structures and are regulated depending on the physiological situation

1.2.4 Connexins of the gap junctions and intercellular communication implication

GJ channels allow direct communication between neighboring cells by providing a passage for ions, cAMP, InsP$_3$, adenosine, glutamate, glutathione, ADP and ATP [29, 30]. Hepatocytes of the liver express Cx32 and Cx26 to some extent, and their GJs channels allow the diffusion of these metabolites and signaling molecules [31]. Oocytes of the female reproductive system require GJs of Cx43 and Cx37 for their oogenesis and proper growth [32]. In male reproductive system, Cx43 is expressed in the testis and plays a role in the gonad development and spermatogenesis [33]. The endothelial cells GJ channels of Cx40 facilitate the propagation of vasomotor signals [34]. In the immune system, Cx43 immunoreactivity between Langerhans cells and T lymphocytes have been detected, signifying the potential role of GJs channels in

antigen presentation, and/or lymphocyte proliferation [35]. In other instances, connexins can be used as biomarkers for pathological conditions. The amount of Cx43 and Cx32 is decreased in the mucosa of the gastric chronic ulcer, and they return to normal levels after antiulcer treatments [36]. In the respiratory system, GJs have been assumed to be rare and not expressed along the air conducting system and the respiratory epithelium. In contrast, GJs play a role in lung functioning and pathology. Recently, in the respiratory epithelium, Cx26, Cx30.3, Cx32 and Cx40 showed a moderate expression when using immunohistochemistry whereas Cx43, Cx46 and Cx32 gave a strong immunoreactivity [37]. Changes in the expression patterns of these respiratory connexins are indication of alveolar injury, type-II cells, or increasing cell proliferations [38]. In the retina, GJ channels are numerous and can be observed in every major retinal cell type, which provide primary signaling pathways between these cell types. There are four neural connexins expressed by retinal cells, and Cx36 is the most dominant of the four (Cx36, Cx45, Cx50 and Cx57) [39]. Lastly, because reduced GJIC ability leads to cell proliferations, it has been hypothesized that the protein connexins work as tumor suppressor [40]. GJ channels serve different functional purposes depending on their location in the specific tissue and organ.

1.2.5 Human diseases and connexins mutations

Aberrations in connexin expressions have been connected to cancer, cardiac ischemia, and cardiac hypertrophy. There are numerous connexins mutations that are linked to inherited human diseases. Point mutations located on TM1, E2 and CL in Cx32 are associated with X-linked Charcot-Marie-Tooth syndrome (CMTX) [41]. Mutations in the Cx47 encoding gene are linked to cause the autosomal recessive

Pelizaeus-Merzbacher-like disease (PMLD) [42] and Hereditary Spastic Paraplegia (HSP), which is a hypomyelinating disease [43]. Deafness and skin diseases are associated with mutations in Cx26 and Cx30 [44]. Mutations in Cx43 are linked to oculodentodigital dysplasia (ODDD), which develops abnormalities in the limb, face, and teeth, but rarely in the heart [45]. Other somatic point mutations in the carboxyl terminal domain of Cx43, which is associated with the phosphorylation of the connexin, appear to be the cause of the heterotaxia (laterality defects) in humans [46] [47]. In addition, mutations in Cx43 related to the phosphorylation sites were found in patients with complex cardiac malformations, but other published reports do not confirm the link [46] [48]. Cataract in humans and mice is associated with mutations located on E1 of both Cx50 and Cx46 [49] [50]. Therefore, it can be concluded that connexins' integrity and proper structure are highly important for the absolute functionality of the gap junction channels.

Functional heterotypic compatibility of Cxs

Cxs	26	32	30	46	50	30.3	37	40	43	45
26	+	+	+	+	+	-	-	-	-	-
32		+	+	+	+	-	-	-	-	-
30			+	+	+	+	+/-	+	+/-	+/-
46				+	+	+	+	-	+	-
50					+	+	-	-	-	
30.3						+	+	+	+	-
37							+	+	+	+
40								+	+/-	+
43									+	+
45										+

Group 1
Group 2

inter-group
intra-group
+ compatible
- no functional channel
+/- conflicting data reported
blank no data

Table. 1.1. Ten well-studied connexins and their compatibility in forming heterotypic GJs

 The ten connexins are divided into two groups, Group1 and Group2. Most intra-group connexin combinations (red boxes) are compatible and can form functional heterotypic gap junctions (GJs). The blue boxes represent inter-group of connexins that, in most cases, cannot form functional heterotypic GJs. The negative sign (-) indicates incompatibility and the positive sign (+) shows heterotypic compatibility in forming GJs (Table. 1.1 is modified from Bai and Wang, 2014). Cx40 and Cx43 compatibility is circled. The combination of these two connexins is labeled with both signs (-/+) indicating conflicting reports with respect to their compatibility.

1.3 Electrical propagation in the mammalian heart

The human heart contracts about seventy times per minute, or more than three billion times in a regular human life span. Normal contraction of the atria and the ventricles within the mammalian heart are completely dependent on the perfect synchronization of the cardiomyocytes in these two chambers. The electrical signal of the heartbeat is initiated mainly by the active pacemaker cells of the sinoatrial node (SA), located in the right atrium close to the junction point of the vena cava. The action potentials are propagated rapidly through GJs located at the intercalated discs between cardiomyocytes of the mammalian heart [51, 52]. The GJ channels in the heart ensure rapid conduction of action potentials in atria and ventricles, synchronizing the contraction at each chamber. From the active pacemaker cells of the SA node, the action potential signal is propagated to the crista terminalis with a velocity of 0.5-1.0 m/s to the right and left atria [53]. Thereafter, the action potentials travel through the conduction system in the following order: the atrioventricular (AV) node which is the sole connecting bridge between the atria and the ventricles, bundle of His, bundle branches, and Purkinje fibers, with some delay at the AV node to ensure that the ventricles contract after the atria contraction (pumping blood into ventricles). After reaching and entering the His bundle, the the action potential propagates and accelerates quickly to 2.0 m/s and stays constant throughout [53]. The AV node also protects the ventricle from the spread of atrial tachyarrhythmias by filtering the high atrial frequencies that occur during atrial fibrillation (AF) [54]. The propagation of action potentials through the chambers of the heart is governed by the tissue architecture, the gap junction channels and other ion channels determining cell

excitability. Connexin isoforms that form the GJs determine the electrophysiological properties of the cardiac GJ channels.
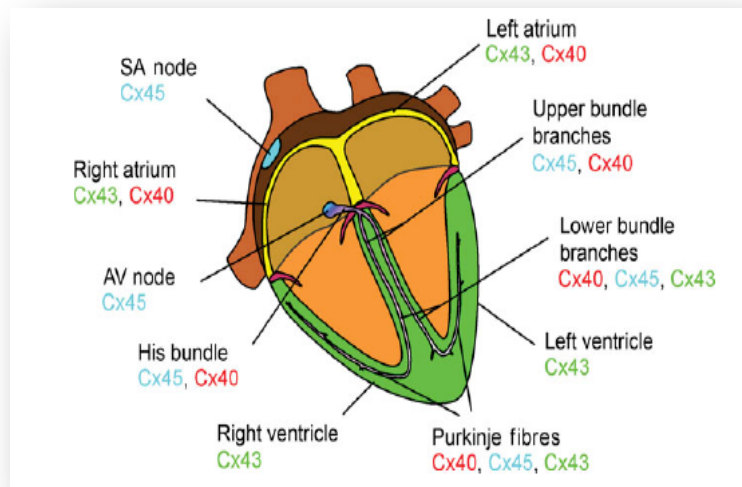
Fig. 1.3. Summary of typical connexins expression patterns in mammalian heart modified from (Severs et al 2008) [55]

Working cardiomyocytes of the atria express Cx40 and Cx43 at similar levels. SA and AV nodes express Cx45 abundantly. The cardiomyocytes of the ventricles express Cx43 predominately. The distribution of Cx40, Cx43 and Cx45 varies from area to the adjacent area in the mammalian heart tissue.

1.4 Connexins of the mammalian heart

There are three major connexins expressed in the mammalian heart. Cx43, the predominant connexin, forms GJ channels at the intercalated discs between the cardiomyocytes of the atria and ventricles as well as the Purkinje fibers [56]. Cardiomyocytes of the SA and AV nodes express Cx45 only as the major component of its GJs, and in the conduction system [57, 58]. Atrial myocytes express both Cx40 and Cx43 equally. Both atrial and ventricular myocytes express low levels of Cx45 and the atria contain marginally higher amounts of Cx45 than the ventricular myocytes [59]. Cardiac connexins show remarkable spatial distributions which contribute to the specific function of the GJs.

Null mutant mice of the cardiac connexin genes have been generated to investigate the role of connexins in cardiac impulse propagation. Deletion of the Cx43 gene resulted in neonatal death due to defect that was confined to the right ventricular outflow tracts [60]. Still, when Cx43 gene is conditionally inactivated in cardiomyocytes solely, Cx43 (CKO) mice develop uniform sudden cardiac death at age of two months due to ventricular arrhythmias [61]. However, Cx40-deficient mice presented conduction abnormalities with slower conduction velocity in the atria, slower conduction in the AV-nodal region, damaged conduction in the bundle branches, and an increased susceptibility to inducible atrial arrhythmia [62]. These experimental findings propose a role of Cx40 and Cx43 in the development of cardiac arrhythmias.

Abundant studies emphasize the importance of GJs in the genesis of cardiac arrhythmias, which has motivated the search for antiarrhythmics aiming regulating GJ

channel function [63, 64]. Some forms of arrhythmia such as atrial fibrillation (AF) appear to be genetically concurrent with somatic mutations of Cx40 [65]. Knockdown-Cx40 mice during adult ages are feasible due to slower atrial conduction velocity and are more susceptible to atrial arrhythmia [66]. Other germ-line autosomal dominant mutations (Q49X, I75F, P85I, L221I and L229M) in Cx40 gene have been found in individula AF patients [67]. One of these mutations (Q49X) was found to cause the disappearance of the GJ plaques that are found at the cell-cell interfaces [68]. Other mutations such as I75F, P85I, L221I and L229M were studied and found to have not affect the ability of the GJ plaques being formed at the cell-cell boundaries, but the coupling conductance (Gj) was severely reduced in heterotypic GJs with Cx43 [69]. Transgenic mutant mice generated with the associated AF mutation (Cx40A96S) exhibited a reduction in atrial conduction velocities, and prolonged intervals of induced AF [70]. In addition, studies strongly linked Cx40 polymorphisms to enhanced AF susceptibility [71]. Correspondingly, sporadic somatic mutation and germ-line mutations in the Cx40 gene were detected in idiopathic atrial fibrillation (AF) patients [65]. Abnormal expression of Cx43 is also often found to be associated with abnormal conduction and arrhythmias [61]. More than 70 mutations in Cx43 gene are linked to oculodentodigital dysplasia (ODDD), which is an autosomal-dominant human disorder. ODDD patients exhibit congenital craniofacial deformities and deficiencies in their eyes, teeth, fingers, and skin [72]. It was a little surprising that no consistent cardiac phenotype was found to be associated with ODDD-linked Cx43 mutants.

1.5 Connexins of the atria

There are several connexin isoforms expressed in the human heart and within its conducting system. However, as mentioned above only two major connexins— Cx40 and Cx43— **(Figure 1.3)** are prominently expressed by the working atrial cardiomyocytes. Cx43 is expressed predominantly by both the atrial and the ventricle cardiomyocytes, while Cx40 is expressed highly and only by the working contractile myocytes of the atria [59]. Since atrial cells could potentially express Cx40 and Cx43 equally, heteromeric and/or heterotypic GJs are likely formed. Researchers have studied the interaction of these two connexins (Cx40 and Cx43) using biochemical analyses such as co-immunoprecipitations and determined the co-precipitation of Cx40 and Cx43 in the atria [73]. However, It is still unknown whether the co-expression of Cx40 and Cx43 in vivo is heteromeric or heterotypic.

1.6 Hypothesis

We hypothesize that critical amino acid residues located on either E1 or E2 domains of Cx40 influence the ability to form functional Cx40/Cx43 heterotypic gap junction channels.

1.6.1 Rationale

The first crystal structure of GJ channels was revealed in 1999 of Cx43 at a resolution of 7.5 Å. Thereafter, in 2009 a crystal structure of high resolution (3.5 Å) of a Cx26 GJ channel was obtained. This high-resolution structure shows very detailed arrangements at the docking interfaces of the opposing hemichannels. At the docking cell-cell interfaces of the GJ channels, there were 60 hydrogen bonds (HBs) at both the extracellular loops, E1 and E2. Subsequently, Cx26 and Cx32 heterotypic GJ was studied in terms of revealing its docking mechanisms. A homology model of Cx32/Cx26 heterotypic GJ channel was created. According to the homology model of Cx32/Cx26, there are specific amino acids residues responsible for these HBs and these are located mainly on the E2 domains. Consequently, sequence alignment of the E1 and E2 domains of Cx40, Cx43, and Cx26 were aligned and the alignments were numbered according to Cx40. The amino acid residues that have been identified in Cx26 as HB-forming residues were localized at specific positions of Cx40 and Cx43. Due to the possibility of the contribution of these residues in Cx26 in the docking mechanism of homotypic and heterotypic GJ, the analogous residues on E1 and E2 have been proposed to be responsible for the docking interaction between Cx40 and Cx43.

1.7 Objectives

1- **Aim 1:** To design three variants according to sequence alignment of Cx40, Cx43 and Cx26: (D55N, P193Q and N195D)

2- **Aim 2:** To examine the localization and the capability to form GJ plaque structures at the cell-cell interfaces of the newly designed Cx40 variants, D55N, P193Q, and N195D. The probability of forming homotypic and heterotypic (with wildtype Cx43 and Cx40) morphological GJ plaque structures will be quantified in HeLa and N2A cells.

   Methods: In order to visualize the localization of Cx40 and its variants, HeLa and N2A cells will be transiently transfected with Cx40-YFP, D55N-YFP, P193Q-YFP and N195D-YFP to study their ability to form homotypic GJ plaque-like structures. Their ability to form morphological heterotypic GJs will be studied using Cx43-mRFP.

3- **Aim 3:** To measure the GJ coupling conductance (Gj) of these Cx40 variants expressed in N2A cell pairs forming homotypic GJ channels or heterotypic GJ with wildtype Cx43 or Cx40 using a dual whole patch clamp technique.

   Methods: Dual whole-cell patch clamping will be used to examine the gap junction function of homotypic channels formed of either Cx40, D55N, P193Q or N195D in N2A cells expressing these constructs. To examine the function of heterotypic channels with Cx43, N2A expressing these Cx40 mutants will be intermixed with Cx43-mRFP expressing cells. Both fusion fluorescent protein

tagged and untagged constructs (using mutant-IRES-GFP and Cx43-IRES-DsRed) will be used to the functional outcome of the heterotypic GJ channels.

4- **Aim 4:** To study transjunctional voltage-dependent gating (Vj-gating) properties of tagged and untagged Cx40 variants in homotypic GJ channels and heterotypic GJ channels with Cx43 in N2A cells.

Method: The techniques of Aim 4 will be the same as those used in Aim 3.

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Chapter 2: Article

Engineered Cx40 variants increased docking and function of heterotypic Cx40/Cx43 gap junction channels

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2.1 Abstract

Gap junction (GJ) channels provide low resistance passage for rapid action potential propagation in the heart. Both connexin40 (Cx40) and Cx43 are abundantly expressed in and frequently co-localized between atrial myocytes, possibly forming heterotypic GJ channels. However, conflicting results have been obtained on the functional status of heterotypic Cx40/Cx43 GJs. Here we provide experimental evidence that the docking and formation of heterotypic Cx40/Cx43 GJs can be substantially increased by properly designed Cx40 variants where the extracellular domains (E1 and E2) have been modified. Specifically, Cx40 D55N and P193Q; substantially increased the probability to form GJ plaque-like structures at the cell-cell interfaces with Cx43. More importantly the coupling conductance (G_j) of D55N/Cx43 and P193Q/Cx43 GJ channels are significantly increased from the G_j of Cx40/Cx43. Our homology models indicate the electrostatic interactions and surface structures at the docking interface are key factors preventing Cx40 from docking to Cx43. Improving heterotypic G_j of these atrial connexins may be potentially useful in improving the coupling and synchronization of atrial myocardium.

Abbreviations Cx40, connexin40; Cx43, connexin43; E1, the first extracellular domain; E2, the second extracellular domain; GJ, gap junction; $G_{j,ss}$, normalized steady-state junctional conductance; I_j, macroscopic junctional current; V_j, transjunctional voltage;

2.2 Introduction

Gap junction (GJ) channels are intercellular channels that provide a direct electrical and metabolic passage between adjacent cells to synchronize many physiological activities. Each gap junction channel is formed by docking of two hemichannels at their extracellular domains. Hemichannels are homo- or hetero-oligomeric proteins of 21 homologous connexins in human [1, 2]. All connexins are predicted to share similar structural topology with four transmembrane domains linked by the first and the second extracellular loop domains (E1 and E2, respectively) and one cytoplasmic loop with both amino-terminus and carboxyl-terminus residing in the cytosol [1, 3]. Both connexin40 (Cx40) and Cx43 are abundantly expressed in the human cardiovascular system, including the atria and the ventricular conduction system of the heart [4-7] as well as the endothelial/smooth muscle cells in the vasculature [8, 9]. In the atria of the heart, Cx40 and Cx43 are co-expressed and frequently co-localized, which give the possibility of forming heterotypic GJ channels [7, 10, 11]. However, conflicting results were obtained on whether Cx40 hemichannels are capable of docking with Cx43 hemichannels to form functional heterotypic Cx40/Cx43 GJ channels [9, 12-16]. Irrespective to the differences in the detailed experimental conditions and the expression model cells used, all of these studies are consistent on that the GJ coupling conductance (G_j) of the heterotypic Cx40/Cx43 GJs is much lower than that of the homotypic GJs containing Cx40 or Cx43. The probability of observing coupling at heterotypic Cx40/Cx43 GJs are from as high as their homotypic GJs [12, 13], less than half [17], or to a very low level indistinguishable to the background coupling of the expression system [9, 14]. The

mechanisms of low efficiency in forming functional heterotypic GJ channels are unknown, partially due to the lack of high resolution structural information for both Cx40 and Cx43 [18].

The first high resolution (at 3.5 Å) crystal structure of human Cx26 GJ channel was resolved in 2009 revealing a total of 60 hydrogen bonds (HBs) at the docking interface between two hemichannels [19, 20]. Twenty-four of them are found between docked E1-E1 and 36 HBs are located at the E2-E2 docking interface [19, 20]. Mutations on the E2 docking HB-forming residues in Cx26 and Cx32 (a docking-compatible connexin to Cx26) are predicted to reduce the number of inter E2-E2 HBs, and often lead to impairment in forming functional homotypic and heterotypic GJ channels [21, 22]. Restoration of the lost HBs at the E2 docking interface in the Cx26 and Cx32 mutants resulted in rescuing the docking and formation of functional GJ channels [21]. These results indicate that the HBs at the docking interface of E2 play an important role in docking and formation of functional homotypic/heterotypic GJ channels in these connexins.

It is well documented that both Cx40 and Cx43 are docking incompatible to Cx26 [23], but it is not clear if Cx40, Cx43 and other docking incompatible connexins also use HBs at the docking interface of their homotypic and heterotypic GJs. Sequence alignment of Cx40 and Cx43 with Cx26 at the E1 and E2 domains showed many conserved amino acid residues, including triple cysteines in both E1 and E2 forming intra-subunit disulphide bonds [20, 24]. The docking HB-forming residues in E1 and their equivalent residues are well conserved, but the HB-forming residues in E2 and their equivalents are not conserved which could alter their ability to form HBs at

the docking interface (Fig. 2.1). We **hypothesize** that Cx40 and Cx43 have a similar structure at the docking interface comparing to that of Cx26 and they use similar residues (equivalent to those HB-forming residues in Cx26) for their docking. Key differences in these putative docking residues between Cx40 and Cx43 prevent efficient docking and formation of functional heterotypic GJ channels. Our homology structure models predict that electrostatic repulsion and docking surface structural differences between Cx40 and Cx43 are important factors preventing the docking and formation of functional heterotypic Cx40/Cx43 GJ channels. Accordingly, we predicted that single missense variants in Cx40 E1 and E2 domains (Fig. 2.1) are able to significantly increase the formation of GJ plaque-like structures and the coupling conductance (G_j) with Cx43. Increasing heterotypic coupling between Cx40 and Cx43 might improve atrial myocytes coupling and synchronization, which could be useful in controlling atrial arrhythmias. Part of this study was published as an abstract [25].

2.3 Results

2.3.1 Sequence alignment of Cx40, Cx43 and Cx26 at E1 and E2

Cx40 and Cx43 are homologous to Cx26 with an overall protein sequence identity at 44.6% and 44.7%, respectively. We aligned part of the E1 and E2 domains of Cx40 and Cx43 with those of Cx26 (Fig. 2.1). The alignment showed high sequence identity, suggesting that Cx40 and Cx43 may share similar structures in these domains as those of Cx26. Looking further into the sequence logo for these domains from all the human connexins capable of making functional GJ channels revealed many well-conserved residues (large letters in the logos, Fig. 2.1), indicating they might be important for GJ structure and function. The hydrogen bond (HB)-forming residues at the docking interface of Cx26 GJ channel are indicated (arrows in Fig. 2.1 A and B) and those on the E2 have been shown to play an important role in docking and formation of functional GJ channels [21, 22]. We hypothesize that the HB-forming residues in Cx26 and their equivalents are important for homotypic and heterotypic docking in Cx40 and Cx43. The differences in one or more of these residues between Cx40 and Cx43 influence their heterotypic docking efficiency. Based on the sequence alignment and the logo, we designed three point mutations in Cx40, D55N, P193Q and N195D (circles in Fig. 2.1A and B). D55N was selected since the equivalent residues among all the connexins are a well-conserved non-charged Asn (N), but in Cx40 a negatively charged Asp (D) is found. For the same reason, we generated the mutant N195D. The position P193 is not well-conserved among connexins. In Cx40 proline (P193) has a non-polarized circle side chain restricting its main chain peptide bond angle, but at the corresponding position in Cx43 is a Gln (Q), which has a long

polarized side chain with more flexibility on the peptide bond angle. These point variants in Cx40 are likely to alter either the electrostatic interactions (D55N and N195D) or the structure (P193Q) of the docking interface, which could improve the heterotypic docking of these two connexins.
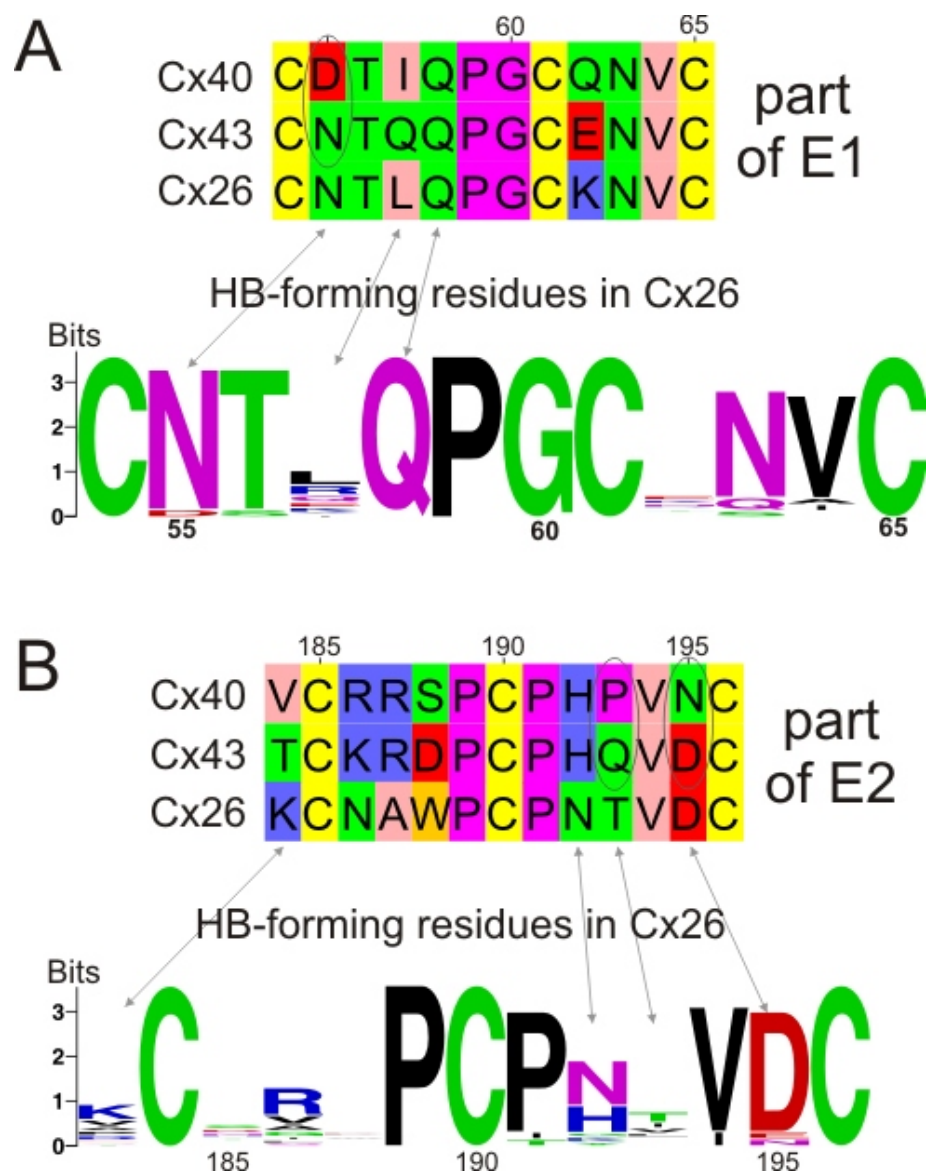
Fig. 2.1. Sequence alignment of the extracellular domains of Cx40 and Cx43 with Cx26

Part of the E1 and E2 domains of Cx40 and Cx43 are aligned with that of Cx26. The docking hydrogen bond (HB)-forming residues in Cx26 structure and their equivalent residues in other connexins are indicated with arrows. Sequence logos were generated with WebLogo [26] for all human connexins capable of making functional GJ channels. Out of the seven putative docking residues, Cx40 D55, N195 and P193 are uniquely different from Cx43 (gray circles) and many other human connexins and are chosen to generate the mutants, D55N, N195D and P193Q.

2.3.2 The localization of Cx40 variants and their co-localization with Cx43 and Cx40

To facilitate the study of the localization of Cx40 variants, YFP-tagged variants were generated and expressed in gap junction deficient HeLa cells. D55N-YFP and P193Q-YFP were localized in the intracellular compartments as well as at cell-cell interfaces forming putative GJ plaques, similar to that of Cx40-YFP (Fig. 2.2). However, N195D-YFP was diffusely distributed in the cytosol and failed to form any GJ plaques at the cell-cell interfaces (Fig. 2.2), indicating that this mutation impaired the steady-state localization. The probability of observing GJ-plaques at the cell boundary in transfected HeLa cell pairs are summarized (Fig. 2.2). Both D55N and P193Q showed similar ability to form GJ plaque-like structures as that of Cx40. Virtually identical observations were obtained using N2A cells as the expression vehicle (open bars in Fig. 2.2).

To study the co-localizations of Cx40 variants with Cx43, Cx43-RFP-expressing HeLa cells were co-cultured with the variant-YFP-expressing cells. Live-cell fluorescent imaging indicated that wildtype Cx40-YFP was unable to co-localize with Cx43-RFP at cell boundaries (Fig. 2.3). However, D55N-YFP was abundantly co-localized with Cx43-RFP at the cell-cell interface forming yellow GJ plaque-like structures (indicated by arrows in Fig. 2.3). The probability of observing yellow GJ plaques between D55N- and Cx43-expressing cell pairs was significantly higher than that of Cx40/Cx43 ($P < 0.001$, Fig. 2.3), indicating that D55 in Cx40 plays a role in preventing co-localization of Cx40 and Cx43 at cell-cell interfaces. Interestingly

D55N-YFP was also able to form GJ plaques with wildtype Cx40-RFP (Fig. 2.3). Similarly, the probability of observing co-localized P193Q/Cx43, or P193Q/Cx40, was much higher than wildtype Cx40/Cx43 ($P < 0.05$ and $P < 0.001$, respectively, Fig. 2.3). However, Cx40 N195D was unable to form any GJ plaque-like structures at cell-cell interfaces with Cx43 or Cx40 (data not shown). Similar observations were also obtained using N2A cells and are shown in the bar graph (white bars in Fig. 2.3). Our results demonstrate that a single mutation in either E1 (D55N) or E2 (P193Q) of Cx40 is sufficient to establish morphological heterotypic GJ plaques with Cx43.
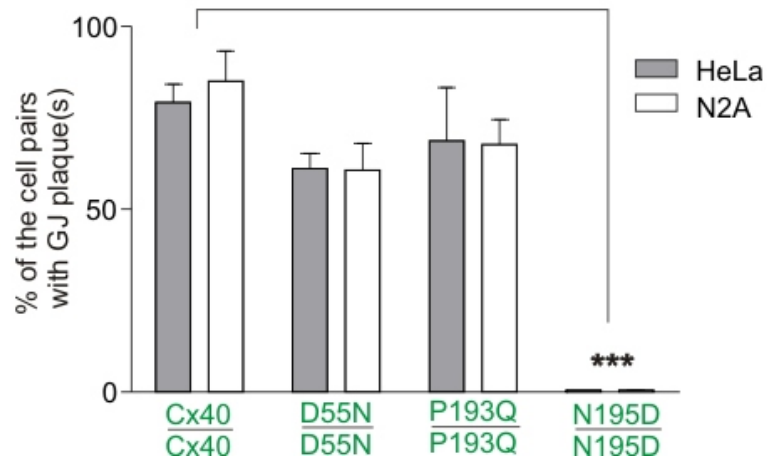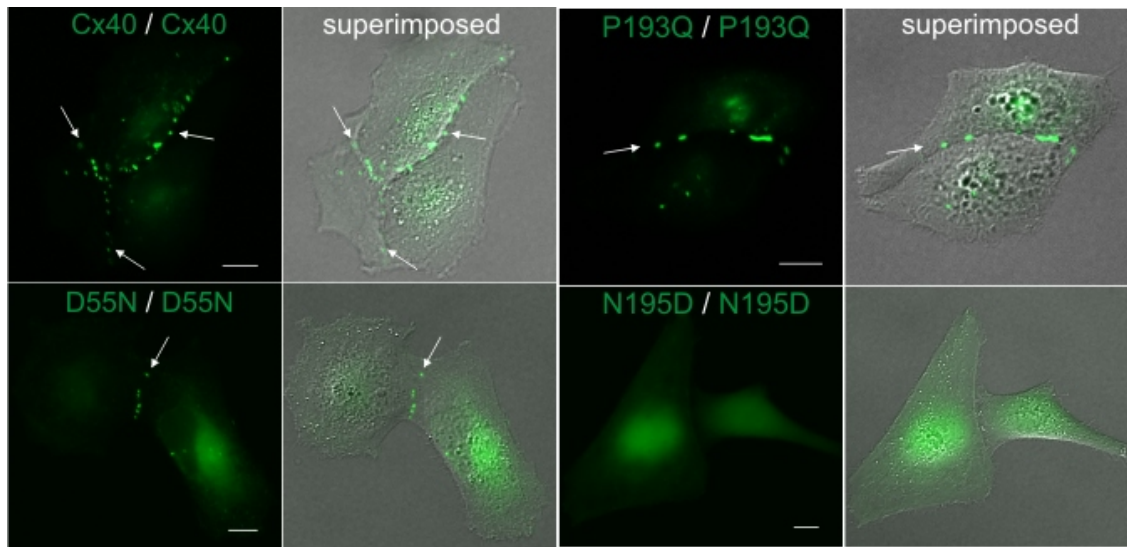
Fig. 2.2. The ability of Cx40 variants to form homotypic gap junction plaque-like structures in HeLa and N2A cells

Fluorescent images (left) and their superimposition on DIC images (right) show paired/clustered HeLa cells expressing YFP-tagged Cx40, D55N, P193Q and N195D. D55N and P193Q were able to form GJ plaque-like structures at the cell-cell interfaces similar to that of Cx40, while N195D was diffusely distributed in the cytosol and failed to form GJ plaque-like structures. Bar graph summarizes the probability of observing GJ plaques in HeLa (gray bars) and N2A (white bars) cells positively expressing the respective constructs. Data are average of 3-4 independent experiments.
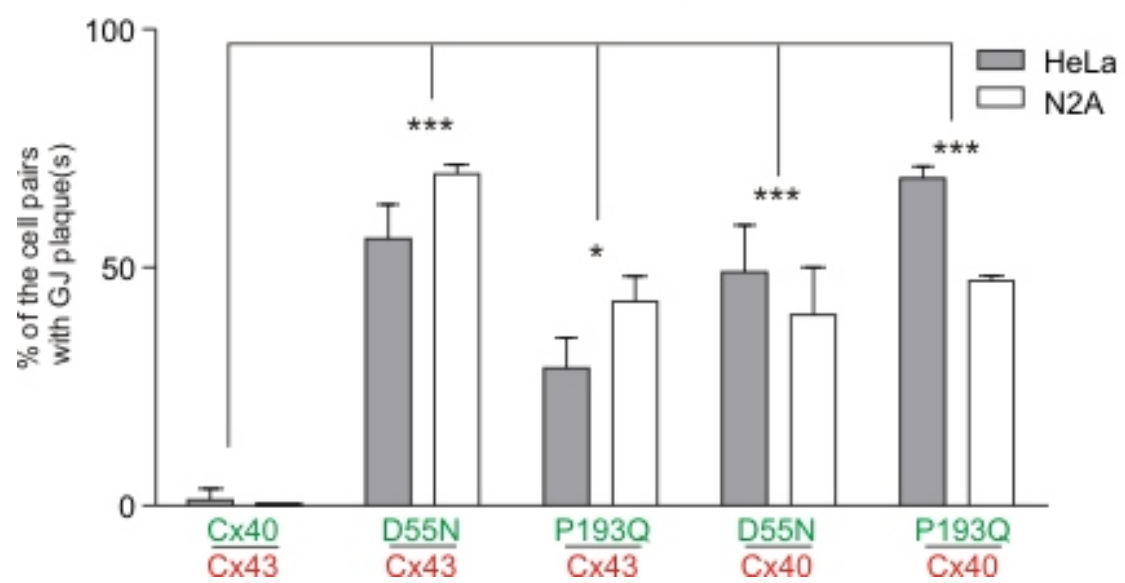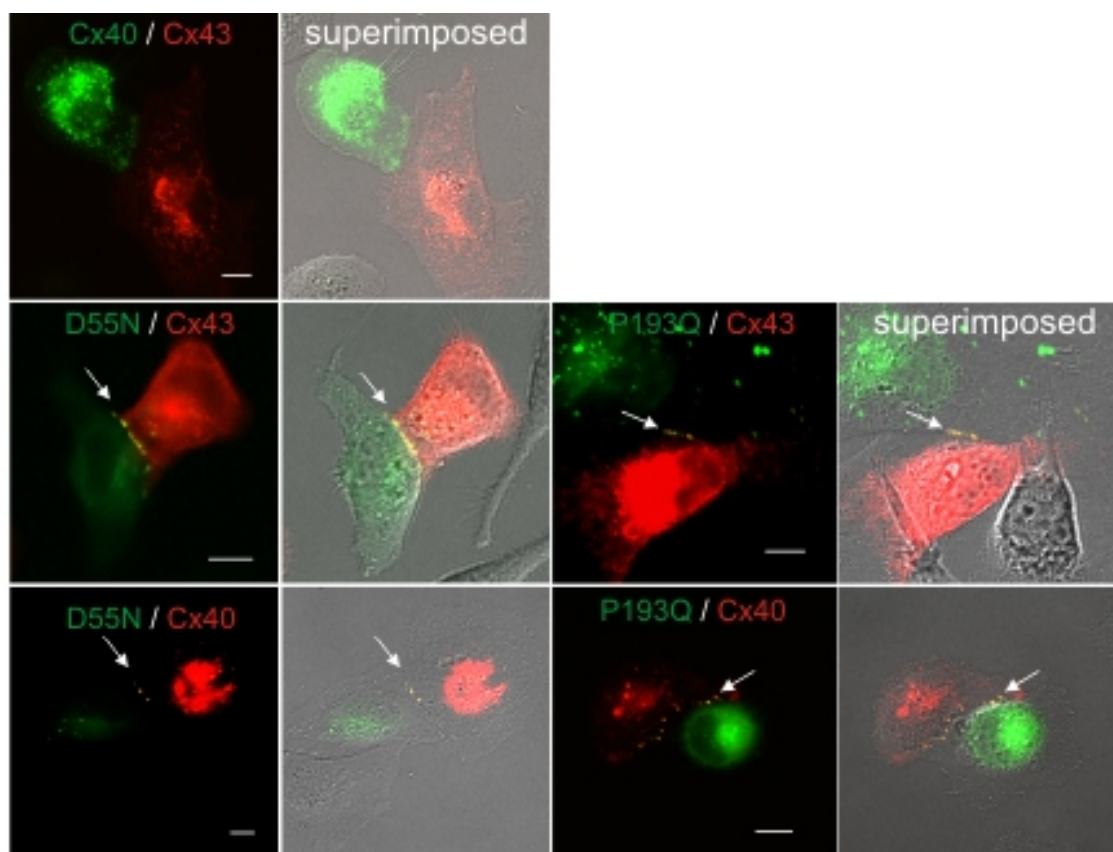
Fig. 2.3. The ability of Cx40 variants to form heterotypic gap junction plaque-like structures in HeLa and N2A cells

Superimposed green (YFP) and red (RFP) fluorescent images (left panels) and their overlay on DIC images (right panels) to show paired HeLa cells successfully expressing Cx40 variant-YFP in one cell and Cx43-RFP (or Cx40-RFP) in the other. At the cell-cell interfaces of Cx40/Cx43 cell pairs, no co-localized (yellow) GJ plaque-like structures were observed. However, in cell pairs expressing D55N/Cx43 or P193Q/Cx43, co-localized yellow GJ plaques were readily identifiable (arrows). Both D55N and P193Q were also able to co-localize with Cx40-RFP at the cell interfaces. Bar graph summarizes the probability of observing heterotypic GJ plaques in 3-4 independent experiments on the HeLa (gray bars) and N2A (white bars) cells positively expressing the respective constructs.

2.3.3 Homotypic gap junction function of Cx40 variants

Functional status of these YFP-tagged Cx40 variants was studied using dual whole-cell patch clamp technique. N2A cell pairs successfully expressing D55N or N195D showed GJ coupling in 4/15 or 0/8 cell pairs, respectively. The coupling probabilities (27% or 0%, respectively) and the coupling conductance ($G_j = 0.3 \pm 0.2$ nS and 0 nS, respectively) were both statistically lower than the cell pairs expressing wildtype Cx40 (15/18 or 83%, see Table S1 and $G_j = 17.1 \pm 3.9$ nS, Fig. 2.4). However, the coupling probability (7/13 or 54%) and the G_j (9.2 ± 4.8 nS) of P193Q were not statistically different from those of wildtype Cx40 (Fig. 2.4). RFP-tagged Cx43 showed a similar coupling% and G_j with those of Cx40 ($P > 0.05$, Fig. 2.4).
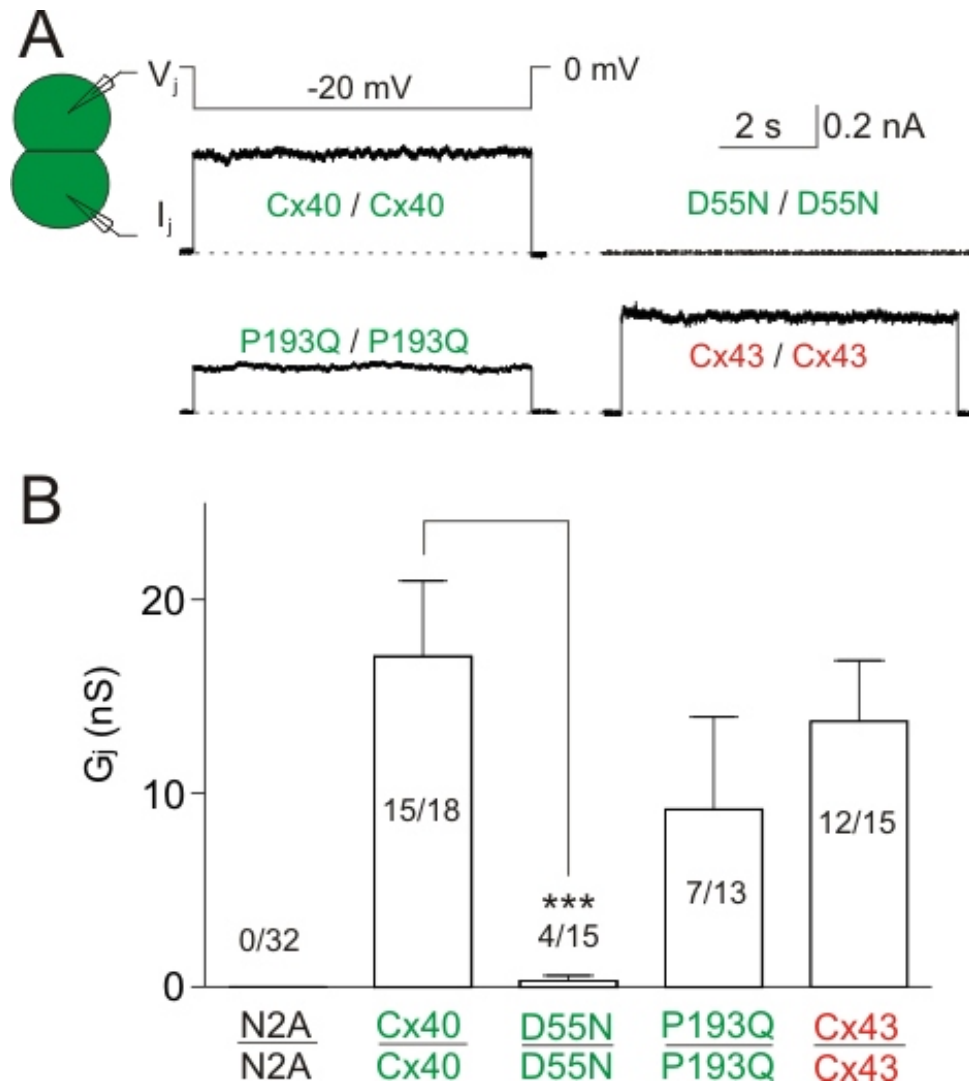
Fig. 2.4. Functional status of homotypic gap junction of Cx40 variants

Dual patch clamp recording was used to measure transjunctional current (I_j) from N2A cell pairs expressing Cx40, D55N, P193Q or Cx43. (B) Bar graph is illustrated on the transjunctional coupling conductance (G_j) of homotypic GJ channels formed in cell pairs expressing YFP-tagged Cx40, D55N or P193Q, as well as RFP-tagged Cx43. Cx40, P193Q and Cx43-expressing cell pairs showed prominent coupling, while cell pairs expressing D55N showed a significantly lower G_j than that of Cx40 (P < 0.001). Untransfected N2A cells were used to serve as negative controls for these experiments.

2.3.4 Heterotypic gap junction function of Cx40 variants with wildtype Cx43 and Cx40

Seven out of twenty-eight cell pairs (25%) with one expressing Cx40 and the other expressing Cx43 showed GJ coupling (Fig. 2.5). The coupling probabilities of D55N/Cx43 (23/28 or 82%), P193Q/Cx43 (14/20 or 70%) and P193/Cx40 (10/11 or 91%) were significantly higher than Cx40/Cx43 (Table S1), but not for D55N/Cx40 (5/12 or 42%). The average G_j of Cx40/Cx43 pairs were extremely low ($G_j = 0.06 \pm 0.02$ nS). Both the G_js of D55N/Cx43 (8.8 ± 2.7 nS) and P193Q/Cx43 (3.6 ± 1.3 nS) were significantly higher than that of Cx40/Cx43. The G_j of P193Q/Cx40 was also significantly larger (13.5 ± 3.8 nS, $P < 0.001$), while the G_j of D55N/Cx40 (5.8 ± 3.2 nS, $P > 0.05$) was similar to that of Cx40/Cx43.
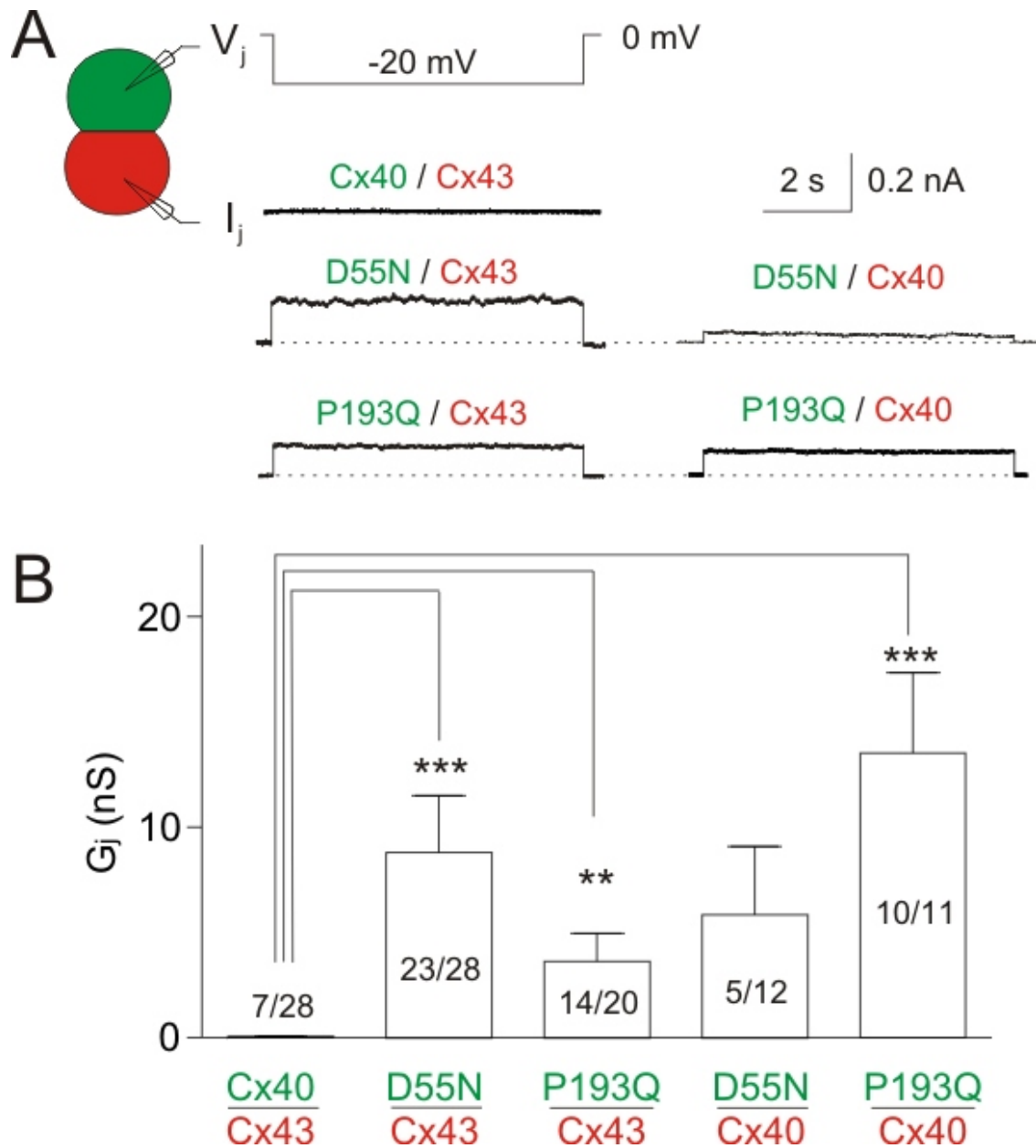
Fig. 2.5. Functional status of heterotypic gap junctions of Cx40 variants

(A) Dual patch clamp recording was used to measure transjunctional current (I_j) from heterotypic N2A cell pairs expressing YFP-tagged Cx40, D55N or P193Q in one and RFP-tagged Cx43 (or Cx40) in the other. (B) The coupling conductance (G_j) of heterotypic GJ channels is shown as bar graph. The G_js of D55N/Cx43, P193Q/Cx43 and P193Q/Cx40 were significantly higher than that of Cx40/Cx43, while the G_j of D55N/Cx40 was not different.

2.3.5 Untagged Cx40 variants showed similar coupling profile with Cx43

Tagging fluorescent proteins at the carboxyl terminus of Cx43 and Cx40 showed little adverse effects on the coupling. To confirm our observed effects of these Cx40 variants were not due to fluorescent protein tagging, we repeated the following experiments with untagged Cx40 variants using IRES-GFP expression vector and Cx43-IRES-DsRed. N2A cell pairs expressing untagged Cx40 showed prominent V_j-gating similar to those observed in tagged Cx40 (Fig. 2.6A). The normalized steady-state to peak ratio ($G_{j,ss}$) against V_j was plotted (open circles for untagged Cx40) and superimposed with that of tagged Cx40 (filled circles). The smooth solid and gray dashed lines represent Boltzmann fitting curves for untagged and tagged Cx40, respectively (Fig. 2.6A, Table S2). As shown in Fig. 2.6A, both the data and fitting curves of untagged Cx40 were virtually identical with that of the tagged Cx40.

Cell pairs expressing tagged heterotypic GJs of D55N/Cx43 showed asymmetric V_j-gating (Fig. 2.6B). When the cell expressing D55N was pulsed to increasingly positive V_j steps (or the Cx43-expressing cell was pulsed to negative V_js), the G_j of D55N/Cx43 channels showed rapid and large deactivation to steady states. However, when the cell expressing D55N was pulsed to negative V_js (or the Cx43-expressing cell was pulsed to positive V_js), the I_j showed very slow and moderate level of deactivation in the tested V_j pulses (Fig. 2.6B). The $G_{j,ss}$ / V_j plot was generated for the tagged D55N/Cx43 channel (Fig. 2.6B). When the D55N-expressing cell was stepped to positive V_j pulses (or the Cx43-expressing cell was stepped to negative V_j pulses), the $G_{j,ss}$ / V_j plot could be fitted with the Boltzmann equation with parameters largely similar to those of homotypic Cx40 channels (Table S2). In contrast, when the D55N-

expressing cell was stepped to negative V_js (or the Cx43-expressing cell with positive V_js), moderate deactivation was observed and the Boltzmann fitting parameters were different from those of homotypic Cx40 GJ channel. Untagged D55N/Cx43 channel displayed similar asymmetric gating. When the Cx43 cell with $-V_j$s substantial deactivation was observed and the Boltzmann parameters are similar to those observed in tagged D55N/Cx43 channels, but the Boltzmann parameters at $+V_j$s (on Cx43-expressing cell) were different (see Table S2). Similar analysis was also performed on tagged and untagged heterotypic P193Q/Cx43 channels and both of the tagged and untagged heterotypic P193Q/Cx43 channels showed similar V_j-gating properties and similar Boltzmann parameters (Fig. 2.6C, Table S2). Our results demonstrated that both untagged D55N and P193Q were also capable of establishing heterotypic docking with Cx43 and forming functional heterotypic GJ channels with comparable V_j-gating properties with those obtained from the tagged variants.
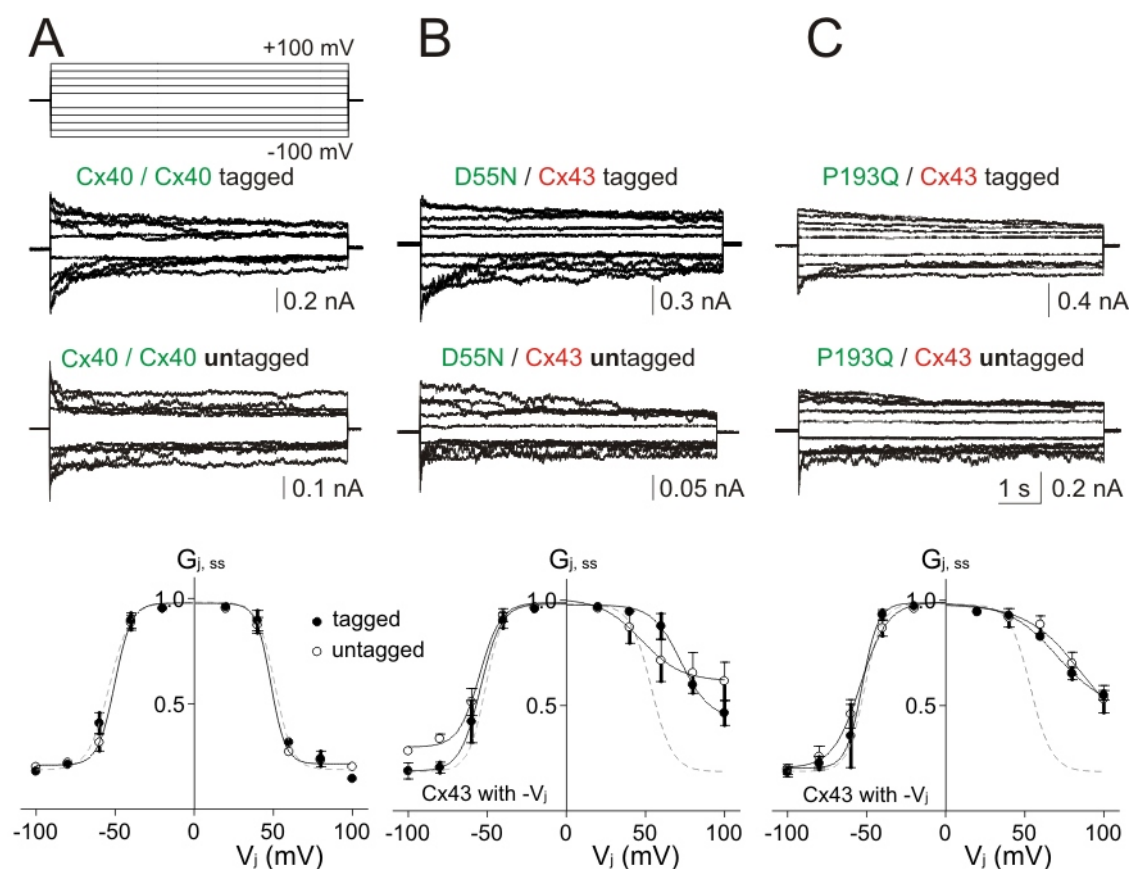
Fig. 2.6. Vj-gating properties of tagged and untagged Cx40 variants with Cx43

(A) Macroscopic transjunctional currents (I_js) from cell pairs expressing tagged and untagged Cx40 were shown in response to the Vj protocol (above). Normalized $G_{j,ss}$ of tagged Cx40 (filled circles) and untagged Cx40 (open circles) were plotted against different V_js. The smooth black lines and gray dashed lines represent the best fitting curves of the averaged data from untagged (n = 5) and tagged (n = 4) Cx40 to a two-state Boltzmann function. (B) I_js were recorded in D55N/Cx43 cell pairs. Both tagged and untagged heterotypic pairs showed similar asymmetric V_j-gating. Boltzmann fitting curves are shown for tagged and untagged constructs. (C) Asymmetric I_js and Boltzmann fitting curves were also observed in P193Q/Cx43 cell pairs. Homotypic Cx40 Boltzmann curves (gray dashed lines) are shown for comparison.

2.3.6 Structural insights on heterotypic Cx40/Cx43 GJ channels

We developed the heterotypic Cx40/Cx43 homology structure model using the Cx26 crystal structure as a template [21, 22]. At the E1-E1 docking interface of Cx43/Cx40 channel model, the negatively charged Cx40 D55 repels electrostatically with two Cx43 residues (Fig. 2.7). The electrostatic repulsions associated with D55 between docked Cx40 E1 and Cx43 E1 could play a role in preventing the docking and formation of functional heterotypic GJ channels. The D55N mutation eliminates the negative charge and is predicted to facilitate the formation of heterotypic D55N/Cx43 channels. In addition, the D55N mutation is unlikely to change the inter E1-E1 HBs (Fig. 2.7). However, a reliable homology model at the Cx40 E2 docking interface could not be achieved since the inclusion of P193 is likely to change the main chain structures, indicating that Cx40 E2 has to have a different structure from those of Cx26 and Cx43. In contrast, the E2 structure of Cx40 P193Q/Cx43 can be modeled with keeping main chain structures of Cx26, we found Q193 forms symmetric HBs with the same residue of Cx43 (Fig. 2.7B), loss of these additional HBs at the E2 docking interface between Cx40 and Cx43 could be an additional factor preventing the docking. Our homology models indicate that electrostatic interactions at E1 and surface structure differences in the E2 are likely mechanisms responsible for the low probability of docking between Cx40 and Cx43. High resolution structures of Cx40 and Cx43 are needed to verify the validity of these models.
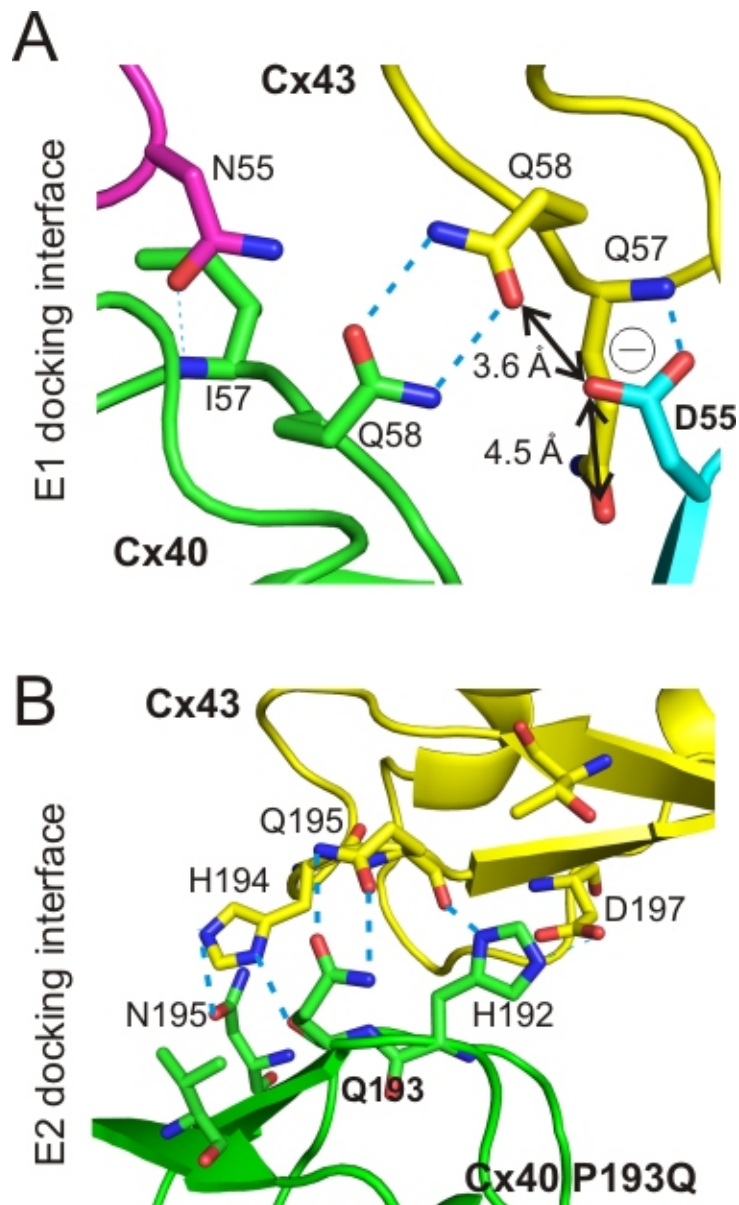
Fig. 2.7 Homology models of Cx40/Cx43 to reveal potential mechanisms responsible for the low docking efficiency

(A) Heterotypic Cx40/Cx43 homology model at the E1 docking interface is shown. Cx40 D55 is negatively charged and close to the carbonyl oxygen groups of Q58 and Q57 on Cx43 (the minimum distances are shown). D55N eliminated the negative charge without changing the inter E1-E1 hydrogen bonds (HBs, blue dashed lines. 4/24 HBs are shown at E1 docking interface). (B) Heterotypic P193Q/Cx43 model at E2 docking interface is illustrated. P193Q mutation made it possible to model the E2 structure based on Cx26. Six inter E2-E2 HBs (blue dashed lines) are predicted to form at the E2 docking interface between each pair of docked E2s.

2.4 Discussion

Consistent with previous studies [9, 14, 17], our present study indicates that Cx40 and Cx43 were unable to form morphological heterotypic Cx40/Cx43 GJ plaques at cell-cell interfaces. In the majority of the cell pairs expressing heterotypic Cx40/Cx43 GJs were not functional. Even if the cell pairs showed function, the G_j was very low. To alter this low probability of heterotypic coupling, we engineered three Cx40 variants according to sequence analysis. Very excitingly, two of our designed variants, D55N and P193Q, were capable of forming prominent morphological and functional heterotypic GJs with Cx43, indicating that both D55 and P193 in Cx40 play a key role in preventing Cx40 from docking efficiently with Cx43. Our homology models indicate that D55 of Cx40 electrostatically repels two glutamine residues (Q57 and Q58) of Cx43 and P193 restricts the Cx40 E2 structure to a conformation incompatible with Cx43 E2. Increasing heterotypic Cx40/Cx43 coupling conductance might be a novel way to augment the GJ coupling and synchronized beatings in atria.

In adult mammalian ventricular myocytes, Cx43 is the dominant connexin making GJ channels. However, in atrial myocytes both Cx40 and Cx43 are abundantly expressed, which potentially make complex types of GJ channels [4-7, 11]. The roles of Cx40 in atrial myocyte coupling and the action potential conduction velocity are not clear. In mouse models of Cx40 gene knockout, conflicting results were obtained. Early studies showed a decrease in action potential conduction velocity and an increase in inducible arrhythmias [27-29]. However, more recent studies indicated an increase in conduction velocity and a decrease in the conduction heterogeneity in the Cx40 knockout mouse [10, 30, 31]. A study on human atrial tissues found that the ratio of

Cx40 / (Cx40+Cx43) was inversely correlated with the conduction velocity [11]. Mutations in human Cx40 gene have been found to link to atrial fibrillation and studies on these mutants in vitro showed functional defects in GJ [32-34] or hemichannels [35, 36]. It is clear from previous studies and this present one that the probability of heterotypic docking of Cx40/Cx43 is low and the resultant GJ coupling conductance is virtually none [9, 14, 17]. It is not clear if this low efficiency in heterotypic docking between these atrial connexins plays a role in decreasing GJ coupling in the atria, which could increase heterogeneity and a higher vulnerability to arrhythmias.

In addition to the heterotypic interaction, Cx40 and Cx43 may interact heteromerically to form heteromeric GJ channels. Several pieces of experimental evidence indicate that these two connexins are likely to oligomerize to form heteromeric channels. First, they are often found to be co-localized [37, 38] and they can be co-immunoprecipitated [8, 39]. Second, the pH-dependent gating was much stronger when Cx40 and Cx43 were co-expressed [40]. Third, multiple single channel conductance levels were obtained when these two connexins were expressed together [8, 13, 39]. Finally, several atrial fibrillation-linked mutants in Cx40 or Cx43 have been shown to have dominant negative actions on the GJ conductance of cell pairs co-expressing these mutants with wildtype Cx43 or Cx40, respectively [32-34, 41]. A structural mutant in Cx40 also showed a similar dominant-negative action on Cx43 [37], indicating that Cx40 is likely to interact with Cx43 to form heteromeric GJ channels. As the theoretical possibilities of forming different stoichiometries of heteromeric Cx40 and Cx43 are large [42], if they do randomly form heteromeric hemichannels and randomly dock to other hemichannels in the neighbouring cells, the

resultant GJ channels are most likely to be heterotypic GJ channels, which are predicted to have low level of coupling based on numerous previous studies [9, 12-14, 17].

All human connexins capable of forming GJ channels are homologous, especially at the E1 and E2 with high protein sequence identity 78% and 57%, respectively [23]. With such a high level of sequence identity to Cx26, it is generally believed that the high resolution crystal structure of human Cx26 GJ channel [19, 20] is an excellent structural template for these GJ channels. With a close inspection on the equivalent docking relevant residues (the docking HB-forming residues in Cx26 E1 and E2 see Fig. 2.1) in these connexins, we found two well-conserved residues (N54 and D179 in Cx26 and their equivalent) are uniquely different only in Cx40 (D55 and N195). At the Cx40 D55 and N195 positions, Cx40 is the only one with Asp (D) at 55th position and with Asn (N) at the 195th position among all the human connexins. To check if these unique sequence variations are only in human Cx40 or in all Cx40 from different species, we searched all available Cx40 sequences from 37 different species (OMA database) [43] and found that all Cx40 at these equivalent positions are conserved [Asp (D) at 55th and Asn (N) at 195th]. It is interesting to learn that Cx40 D55N sufficiently altered the Cx40 E1 electrostatic property to make it compatible in docking with Cx43 to form morphological and functional GJ channels. However, we could not test Cx40 N195D due to impairment in subcellular localization likely due to changes in protein trafficking, degradation and/or the combinations of these processes. Thus, N195 in Cx40 is likely to serve both docking and an additional role in trafficking/degradation pathways.

In summary, our engineered Cx40 variants, D55N and P193Q, substantially improved the heterotypic docking efficiency between Cx40 and Cx43 and both are capable of forming functional GJ channels when docked with Cx43. Our homology models indicate that electrostatic interactions (D55) and structural changes (P193) prevent Cx40 from docking to Cx43. Revealing the detailed structure requirements for the heterotypic docking of Cx40/Cx43 GJ channels might be useful in improving the GJ coupling of atrial tissues.

2.5 Materials and Methods

2.5.1 Plasmid construction

The C-terminal fusion fluorescent proteins tagged human Cx40-YFP, Cx40-RFP (pTag-RFP) and untagged constructs, Cx43-IRES-GFP, were created as previously described [33, 34]. Cx40-IRES-DsRed was generated by subcloning Cx40 from Cx40-IRES-GFP into a pIRES2-DsRed2 vector between the restriction sites, XhoI and BamHI. Cx43-RFP (monomeric RFP) was a gift from Dr. Dale Laird [44]. Cx40-YFP and Cx40-IRES-GFP vectors were used as the templates to generate the tagged and untagged individual point mutants (D55N, P193Q and N195D), respectively, with a Quick-Change site directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used to generate these mutants are as follows:

D55N   Forward: 5' GATTTCCGGTGTAATACGATTCAGCC 3'

Reverse: 5' GGCTGAATCGTATTACACCGGAAATC 3'

P193Q   Forward: 5' CCCTGTCCCCACCAGGTCAACTGTTAC 3'

Reverse: 5' GTAACAGTTGACCTGGTGGGGACAGGG 3'

N195D   Forward: 5' CCCCACCCGGTCGACTGTTACGTATCC 3'

Reverse: 5' GGATACGTAACAGTCGACCGGGTGGGG 3'

2.5.2 Cell culture and transient transfection

Mouse neuroblastoma (N2A) and the human cervical carcinoma (HeLa) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) [33, 45]. Before transfection, cells were plated in 35 mm dishes and the confluence was around 50% after overnight culture. About 1 - 1.5 μg Cx40, Cx43 or one of the mutant vectors was transfected with 2 μl X-tremeGENE HP DNA Transfection Reagent (Roche Applied Sciences, Indianapolis, IN). N2A cells were cultured for 24 hours after transfection and replated onto glass coverslips ~2-3 hours prior patch clamping recording. For V_j-gating study the replating time was reduced to 1-2 hours.

2.5.3 Localization of fluorescent protein-tagged Cx40 and Cx43

The localizations of Cx40-YFP, mutant-YFP, Cx40-RFP and Cx43-RFP were studied by expressing these constructs individually in gap junction deficient HeLa and N2A cells. The cells were replated onto glass coverslips for ~2-3 hours to allow formation of GJs. Red and green fluorescent images, as well as DIC images were obtained from a CCD camera mounted on a fluorescent microscope (Olympus BX51) using a 40x water immersion lens. To show co-localization of different fluorescent protein tagged Cx40 mutants and wildtype connexins at cell-cell interfaces, these fluorescent images and DIC images were superimposed using ImageMaster software. To quantify the percentage of successful formation of GJ plaque-like structures, ~20-60 cells were counted for each transfection.

2.5.4 Electrophysiological recording

The V_j-gating property of cell pairs expressing either Cx40 or its mutants was measured by dual whole-cell voltage-clamp technique as described earlier [45-47]. Briefly, the transfected cells were replated on glass coverslips and then transferred to a recording chamber on an upright microscope (Olympus BX51) filled with extracellular fluid (ECF) at room temperature ($22 - 25^{o}C$). The composition of ECF is (in mM): 140 NaCl, 2 CsCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, 4 KCl, 5 D-(+)-glucose, 2 Pyruvate, pH 7.2. Paired YFP-positive cells were patched by two glass micropipettes (pipette resistance 2 - 4 MΩ) which were filled with intracellular fluid (ICF) containing (in mM): 130 CsCl, 10 EGTA, 0.5 CaCl$_2$, 3 MgATP, 2 Na$_2$ATP, 10 HEPES, pH 7.2. To test V_j-gating of an isolated cell pair, one cell of the pair was clamped at 0 mV while the apposed cell was administrated with a series of voltage pulses (7 seconds in duration) from ± 20 mV to ± 100 mV in 20 mV increments. The junctional currents (I_js) were amplified with the Axopatch 700A amplifier (Molecular Devices, Sunnyvale, CA) with a low-pass filter (cut-off frequency 1 kHz) and digitalized at a 10 kHz sampling rate via an ADDA converter (Digidata 1322A, Molecular Devices, Sunnyvale, CA). Macroscopic GJ conductance (Gj) was calculated with series resistance corrected as previously described [48]. Gap junction impermeable dye, Dextran Texas Red (molecular weight 10,000 Da, 0.25 mg/ml in pipette solution), was sometimes used to ensure some of the highly coupled cell pairs were not due to a cytoplasmic bridge [33]. Numerical calculations were used to correct junctional voltage errors resulting from the series resistance of each patch electrode in the Gj calculations according to [48].

2.5.5 Data analysis

Data are expressed as means ± SEM. Kruskal-Wallis test was used to compare the coupling conductance (G_j) from different homotypic and heterotypic pairs (GraphPad, La Jolla, CA). 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.

To minimize the influence of series resistance on V_j-gating properties, only those cell pairs with ≤ 5 nS junctional conductance (G_j) were selected for Boltzmann fitting analysis [49]. Boltzmann fitting curves were obtained using Graphpad software. For each current trace, the normalized steady-state conductance ($G_{j,ss}$) was obtained by normalizing the steady state current to the peak current. The $G_{j,ss} - V_j$ plots were fitted with a two-state Boltzmann equation for both V_j polarities:

$$G_{j,ss} = (G_{max} - G_{min})/ \{1 + exp[A(V_j - V_0)]\} + G_{min}$$

V_0 is the voltage at which the conductance is reduced by half $[(G_{max} - G_{min})/2]$, G_{max} is the maximum normalized conductance, G_{min} is the normalized voltage-insensitive residual conductance, and parameter A, describing the slope of the fitted curve, reflects the V_j sensitivity of the GJ channels.

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Author contributions

A.J. designed and performed most of patch clamp experiments; analyzed data and wrote an early draft of the manuscript. H.A. participated in the design of Cx40 variants and developed the homology models. H.C. designed and performed part of the localization experiments and related data analysis. W.G.Y. designed and performed some patch clamp experiments on untagged Cx40 variants. D.B. designed the project, supervised data analysis and critically revised the manuscript.

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Engineered Cx40 variants increased docking and function of heterotypic Cx40/Cx43 gap junction channels

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Supplementary Materials

Table S1. Probabilities of homotypic and heterotypic coupling in cell pairs expressing

 Cx40 variants

A: homotypic cell pairs

vector	coupled pairs	pairs not coupled	coupling percentage	different from Cx40[†]	different from N2A[†]
Cx40	15	3	83%		YES***
D55N	4	11	27%	YES**	YES**
P193Q	7	6	54%	NO	YES***
N195D	0	8	0%	YES***	NO
Cx43	12	3	80%	NO	YES***
N2A	0	32	0%	YES***	

B: heterotypic cell pairs

heterotypic pair	coupled pairs	pairs not coupled	coupling percentage	different from Cx40/Cx43[†]	different from N2A[†]
Cx40/Cx43	7	21	25%		YES**
D55N/Cx43	23	5	82%	YES***	YES***
P193Q/Cx43	14	6	70%	YES**	YES***
D55N/Cx40	5	7	42%	NO	YES***
P193Q/Cx40	10	1	91%	YES***	YES***

[†]Fisher's exact tests were performed between each combination with their respective controls (Cx40, Cx40/Cx43, or N2A cell pairs) and the statistical significant difference is indicated YES (**$P < 0.01$ or ***$P < 0.001$) or NO.

Table S2. Boltzmann fitting parameters for homotypic and heterotypic GJ channels of Cx40 variants

vectors	V_j	G_{min}	V_0 (mV)	A
Cx40	+	0.19 ±0.02	53.7±1.3	0.15±0.02
(tagged)	-	0.19±0.02	51.2±1.5	0.18±0.03
Cx40	+	0.22±0.02	48.5±1.2	0.22±0.03
(untagged)	-	0.21±0.02	50.4±1.3	0.19±0.02
D55N/Cx43	+	0.44±0.05**	72.4±3.4**	0.11±0.03
(tagged)	-	0.19±0.04	54.3±2.2	0.15±0.04
D55N/Cx43	+	0.62±0.08**	47.3±11.5	0.08±0.07
(untagged)	-	0.30±0.02**	54.9±1.7	0.15±0.03
P193Q/Cx43	+	0.32±0.04*	82.3±16.6**	0.06±0.04
(tagged)	-	0.20±0.03	54.0±2.1	0.11±0.02
P193Q/Cx43	+	0.48±0.05**	70.5±4.1	0.07±0.01*
(untagged)	-	0.20±0.04	52.8±3.2	0.20±0.07

Data are presented as mean ± SEM and V_0 are absolute values. Student's t-test was used to compare the Boltzmann fitting parameters of the mutants against those of the wildtype Cx40-YFP with the same V_j polarity. The statistical difference levels are shown * P < 0.05 or ** P < 0.01.

Chapter 3: Discussion

3.1 The main points of the study

The present study identified specific amino acid residues that can foster the formation of functional heterotypic Cx40/Cx43 GJ channels. Three variants (D55N, P193Q and N195D) of Cx40 were designed and tested individually for their abilities to form functional heterotypic GJ with Cx43. The functions of homotypic (mutant to mutant) or heterotypic (mutant to wildtype Cx43) GJ channels were investigated using a dual whole-cell patch clamp technique using N2A cells. The ability of these proteins to form morphological GJ plaques at the cell boundaries was examined by the fluorescent microscopy with fluorescently tagged connexin mutants using both N2A and HeLa cells. My results showed that two variants (D55N and P193Q) were capable of forming GJ plaque-like structures in cell pairs expressing homotypic D55N/D55N, P193Q/P193Q, D55N/Cx40, P193Q/Cx40 GJ channels. However, the third mutation (N195D) failed to show any GJ plaque-like structures at the plasma membranes of the apposing cells expressing this mutant. Morphological heterotypic GJ plaque-like structures at the cell boundaries were readily identifiable in cell pairs expressing D55N/Cx43 and P193Q/Cx43 but were absent in cell pairs expressing wild-type Cx40/Cx43. Electrophysiological results showed that gap junctional conductance, G_j, was significantly increased when Cx43 cell was paired up with Cx40-D55N cell compared to when Cx43 cells was paired up with wild type Cx40. Similarly, G_j was also increased in the heterotypic GJ of cell pairs expressing P193Q/Cx43 compared to the G_j of cell pairs expressing Cx40/Cx43. These observations indicate that Cx40-D55N and Cx40-P193Q were able to form functional heterotypic gap junction channels with wild-type Cx43.

3.2 Why were extracellular loop domains chosen to improve the docking interaction over other domains?

The literature states that heterotypic gap junction formations are possible in cell pairs expressing docking compatible connexins, but not possible in cell pairs expressing docking incompatible connexins. For example, homomeric Cx43 connexons can successfully form efficient docking interactions with homomeric Cx30.3, Cx37, Cx45 and Cx46 connexons but not with Cx31, Cx31.3, Cx32, Cx33, Cx40 or Cx50. Homomeric Cx40 connexons are able to dock with Cx37 and Cx45 to form functional homomeric heterotypic channels, but not with Cx31, Cx31.1, Cx32, Cx43, Cx46, or Cx50 [1]. The mechanisms of docking incompatibility between connexins are not fully understood, but have been proposed as relating to the extracellular loop domains. Chimeras with switched extracellular domains were used to study which extracellular domain (E1 or E2) is critical for heterotypic docking in the non-compatible connexins [2] [3]. For example, a chimeric connexin in Cx50 was generated, Cx50E2Cx46, in which the E2 of Cx50 was switched with the E2 of Cx46. This chimera successfully docked with Cx43, a docking incompatible connexin to Cx50, and this successful docking indicates that E2 plays an important role in docking compatibility [2]. In another study, exchanging both the E1 and E2 domains of Cx40 with those of Cx43 allowed the formation of functional heterotypic Cx40/Cx43 GJ channels, suggesting that both E1 and E2 may play a role in docking between Cx40 and Cx43 [4]. Based on these early studies, we aligned the extracellular loops (E1 and E2) of Cx40, Cx43, and Cx26 to identify possible residues responsible for docking compatibility **(Figure 2.1).** In general, both extracellular loops are highly

conserved among connexins, although E2 displayed more sequence variability than E1 [5].

The crystal structure model of Cx26 with a resolution of 3.5 Å and the homology model of Cx26/Cx32 show a total of sixty hydrogen bonds (HBs), which are found at the E1 and E2 interfaces with the docked hemichannels [6] [7] [3]. The total number of inter E1-E1 HBs is 24 or 4 per pair of docked E1s. Similarly, 36 HBs are found between E2-E2 docking interfaces or 6 per pair of docked E2s. Further studies with mutants on the key HB-forming residues in Cx26 and Cx32 indicated that the minimum number of E2 HBs has to be four or higher (per pair of docked E2s) to form functional homotypic and heterotypic GJ channels [8]. The docking mechanisms of other connexins, including Cx40 and Cx43, are unknown, but may use residues equivalent to the E1 and E2 HB-forming residues in Cx26 or Cx32. This was our rationale to generate point mutations (variants) at the Cx40 residues equivalent to the docking HB-forming residues in Cx26. Compared with Cx43 and other connexins, three of these putative docking relevant residues (one is on E1 and two are on the E2 domain) were selected to generate the mutants used in my thesis.

3.3 Why did particular amino acid residue mutations make two different connexons dock and form functional heterotypic GJs?

The specific goals of this study are to determine whether Cx40 and Cx43 can form a functional heterotypic gap junction (GJ) and to characterize the functional properties of these GJs. The most interesting subject of this project is the controversial aspect behind the docking interaction of Cx40 and Cx43 in forming a functional heterotypic GJ.

Several previous studies have suggested the formation of functional heterotypic Cx40/Cx43 GJ channels [9-11]. However, the obtained G_j levels of heterotypic Cx40/Cx43 GJs were low. Perhaps due to the different procedures and different cell types used in these experiments, the measured G_js were not consistent and the probabilities of observing coupling were also different among these studies [9-11]. A few other studies, on the other hand, observed extremely low levels of coupling and low probability of coupling between Cx40 and Cx43 expressing cells, leading to the conclusion that it is impossible to form functional heterotypic GJs between these connexins [12-14].

To visualize the expression and subcellular distribution of Cx40 variants, we generated fused fluorescent proteins at the carboxyl terminus of the mutations and they are identified as tagged (YFP or RFP). To confirm that our results were not affected by the fused protein, we have generated another set of cDNA vectors identified as untagged mutants (via an IRES-EGFP or IRES-DsRed vectors). The answer to the above question of why specific amino acid residues of Cx40 were chosen over others is dependent on the particular type of amino acid and its location. The first variant was chosen because it is an HB-forming residue in Cx26 and located on the extracellular loop 1 (E1) and is named D55N-YFP. At 55 position of Cx40 and equivalent residues, all connexins have a well-conserved amino acid residue, a polar amino acid Asparagine (N). Cx40 is the only connexin at this position with a charged amino acid residue, D (aspartic acid or Asp). The other position, the 193rd amino acid residue on extracellular loop 2 (E2), is selected to generate a variant P193Q-YFP. This position has the hydrophobic amino acid Proline (P) in Cx40 and was chosen because its equivalent in Cx26 is one of the docking HB-forming residues [6]. Proline has a cyclic side chain that might be able to restrict the main peptide

chain of E2 in Cx40 in the three-dimensional structure of the Cx40 docking surface, restricting its ability to dock with Cx43. The other proposed site located on E2 is the 195th position. The equivalent residue of N195 of Cx40 in Cx26 is also involved in the docking HB formation. Nearly all connexins possess an Asp (D) residue at this position, except Cx40, which is Asn (N). Therefore, point mutation (N195D) in Cx40 was generated to test if this residue is responsible for preventing the formation of heterotypic GJ channels with Cx43. Dual whole voltage was used to measure the G_j of the N2A cells expressing homotypic Cx40/Cx40, D55N/D55N, P193Q/P193Q and Cx43/Cx43 GJ channels and heterotypic GJ of D55N/Cx43 and P193Q/Cx43. From the electrophysiological data, cells expressing D55N or P193Q variants when paired with Cx43 expressing cells were able to show prominent GJ coupling with much-elevated G_js compared to that of the wildtype Cx40/Cx43. For the tagged and untagged versions of each mutant, we obtained similar results, confirming the ability of these variants to form functional interactions with Cx43. This study is unique because we were able to identify which amino acid residue when mutated can form functional interactions with Cx43.

3.4 Does the docking incompatibility between Cx40 and Cx43 play a role in reducing the level of GJ coupling in atrial myocardium?

Generally, remodeling of the gap junctions that would affect or change cell-cell communication is considered a viable factor for arrhythmogenesis [15]. Functional properties and relative levels of expression of connexins are thought to influence the GJ coupling levels and the resultant action potential conduction velocity and any alteration in the propagation may prompt cardiac arrhythmias [16-18]. Atrial myocytes express Cx40

and Cx43 abundantly. The endothelial cells of the blood brain barrier (BBB) also express both Cx40 and Cx43 [19]. The endothelial cells of the arteries and veins also express Cx40. Alongside Cx40, Cx43 is expressed by the smooth muscles of the vascular walls [20]. It has been suggested that Cx40 and Cx43 based on GJ coupling are important components to ensure rapid action potential conduction velocity in the atrial myocardium. A single cell that expresses two connexins can form up to fourteen possible homomeric and heteromeric hemichannel configurations. This cell, when paired up to form a GJ channel with another adjacent cell that expresses two connexins, can form nearly 200 different heterotypic and heteromeric GJ configurations [21]. However, the nature of these GJ configurations in the native atrial cardiomyocytes is unclear. In theory, the Cx40 and Cx43 expressed in atrial myocytes can form homomeric homotypic, homomeric heterotypic and heteromeric heterotypic GJ channels. These GJs would have distinct channel properties with different levels of coupling and sensitivity to the transjunctional voltage changes (Vj-gating properties), which could make atrial tissue much more heterogeneous in GJ coupling and possibly alter the action potential propagations. This could be an important contributing factor to explain why atria are much more vulnerable to arrhythmias than ventricles [22]. Heterozygous Cx43 knockout mice showed significant effects in the conduction velocity of the ventricle but not the atrium [23]. In early studies, homozygous Cx40 knockout mice had reduced atrial conduction velocity, whereas heterozygous Cx40 mice revealed a normal conduction velocity [24, 25]. Another study implied the loss of interatrial conduction velocity heterogeneity in heterozygous Cx40 knockout mice [26]. Clinically, somatic and germ-line mutations of Cx40 were found to be associated with the AF incidence [27-29].

3.5 Future studies

My study assessed if the artificial D55N and P193Q mutations formed functional gap junctions using connexin-deficient neuroblastoma cells (N2A). This study was limited by the use of only one cell line. Future studies should use/progress towards atrial cardiomyocytes, as these are more physiologically relevant. Several obstacles could be encountered when dealing with adult atrial myocytes, including low transfection efficiency. Because adult atrial cardiomyocytes are flat and elongated, performing the dual whole-cell patch clamp technique on these cells can be difficult and result in a lower yield.

One possible future study would be to introduce the Cx40 variants capable of docking with both Cx43 and Cx40 into the atria in animal models. These animal models are expected to serve as represent important tools to study atrial fibrillation and can give more detailed information on the outcome these 'super' compatible Cx40 variants have on atrial arrhythmias. Electrophysiological recordings, such as the ECG, can be obtained to monitor atrial arrhythmias.

The promising findings of this study, in fact, open the way for several future projects. First, the variants that were engineered and tested were located on Cx40 only. It would be exciting to have these variants switched over at the same locations in Cx43 and examine whether the results are identical or absolutely inverse. One of the variants is located at 55 where the D amino acid mutated into the N amino acid. Experiments can be conducted on Cx43 mutated at location 55 by having amino acid N switch to D. In vivo

and in vitro studies can be conducted to further examine the behaviors of the mutations of both Cx40 and Cx43.

3.6 Limitations

The study of the docking interactions between Cx40 and Cx43 used the sequence alignments of these connexins with Cx26 and relied on the crystal structure of Cx26. This approach was greatly informative as it revealed some critical docking residues, including the amino acid residues located at the 55th and the 193rd position of Cx40. Our homology structure models are powerful in revealing the structural mechanisms of why Cx40 is unable to dock with Cx43. Ideally, we would like to know the experimentally determined high-resolution crystal structures of either Cx40 or Cx43 to confirm if our homology model predictions are accurate. The availability of these Cx40 or Cx43 crystal models will also help us to design other variants to improve docking or other inter molecular interactions.

One other limitation of this study is the limited knowledge of the co-localization of Cx40 and Cx43 occurs in the human atrium. Atrial myocytes express both Cx40 and Cx43, but there is little known on how they change and regulate the composition of the gap junctions, as any alteration in the propagation of the action potential through the gap junctions can increase the probability of arrhythmia. In turn, modifications in the expression of the Cx40 have been connected to atrial arrhythmogenesis. Improving the coupling of heterotypic Cx40/Cx43 GJ channels could be useful for treatment of atrial arrhythmias. In fact, how the atrial cardiomyocytes govern and regulate the compositions of connexins forming the gap junction channels is not clear. A probability calculation for

the formation of gap junction channels types has been estimated for Cx40/Cx40 and Cx43/Cx43 homotypic GJ channels [30]. Because atrial cardiomyocytes express Cx40 and Cx43 with possibility of detecting 194 different type of GJ, then the probability of observing homomeric homotypic GJ would be 0.024% (that is in case of prevalence of 50% for both connexins). On the other hand, if the prevalence of expression by the atrial cardiomyocytes were shifted towards Cx40 over Cx43, the incidence of observing Cx40 homomeric/homotypic channels would increase to 0.77%, which is 32-fold increase and 130-fold decrease for Cx43 homotypic GJ. Nevertheless, the coupling conductance of heteromeric GJ channels of cardiac connexins (Cx40 and Cx43) was lower than their homotypic counterparts, which suggest that some heteromeric heterotypic Cx40/Cx43 GJ were non-functional [9]. Unlike heterotypic studies, both in vitro and in vivo studies heteromeric GJ channels are non-distinguishable from homotypic and heterotypic GJ channels. In pathological condition such as hypertension, atherosclerosis and ischemia, the regulation of the connexins expression during these disease cases will be dynamically altered. Study in vivo the electrical coupling of atrial myocytes through different type of gap junction channels has not yet been clearly demonstrated.

3.7 Summary

In short, this study aimed to solve the controversial aspect of whether Cx40 and Cx43 can form functional heterotypic GJs and aimed to design specific variants that can make a better docking interaction between these two atrial connexins. The Cx40 variants that were chosen are important residues in heterotypic Cx40/Cx43 docking. The variants were tested using a dual whole-cell patch clamp technique separately and paired with

Cx43. Two (D55N, P193Q) out of three (N195D) Cx40 variants were able to form GJ plaque–like structures at the cell-cell interfaces in HeLa and N2A cells. Functional studies carried out using the two successful variants only and excluding N195D. Using N2A and the patch clamp technique, voltage steps were applied on one cell and the amounts of current passed through the GJs were recorded on the other cell. D55N/Cx43 and P193Q/Cx43 had G_j that was significantly higher than the G_j of Cx40/Cx43 GJ, which is very close to the baseline. In conclusion, we were successful at determining which amino acid residues are responsible for the docking interaction between the two cardiac connexins.

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Curriculum Vitae

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EDUCATION

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Project title: Compatibility of heterotypic Cx40/Cx43 gap junction

B.S   **Wayne State University, Detroit, MI, USA**   Sept 2011-Aug 2013
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WORKING EXPERINCE

Children Caregiver and Provider,   Nov-March 2012
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Statistic and Data Base Analysis (Research Associate)   July- Sept 2012
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RESEARCH EXPERINCE

Undergraduate Researcher   Jan-Aug 2013
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Undergraduate Researcher   Jan-April 2013
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PROJECTS

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GLANSIS ORGANISM RISK ASSESMENT: Filinia Cornuta:

- **Potential For Establishment**

    1- Invasive Biological/Ecological attributes
    2- Environmental compatibility
    3- History of invasion and spread

- **Potential For Introduction**
    1- Potential introduction via dispersal
    2- Potential introduction via hitchhiking/fouling
    3- Potential introduction via unauthorized intentional
    4- Potential introduction via stocking/planting or escape from recreational culture
    5- Potential introduction visa escape from commercial culture
    6- Potential introduction via shipping

- **Potential For Impact**
    1- Potential environmental impact
    2- Potential socio-economic impact

AWARDS

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- Recognition for Academic Achievement of Attaining High
  Scholastic Succeed (**)   ***Wayne State University, MI, USA   2013***
- Dean's List (two years)   ***Wayne State University, MI, USA 2011-2013***
- Ontario graduate scholarship grant (3 term)   ***Western University, London, ON,CA   2013***
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- UWO Physiology & Pharmacology Research Day
  (Winner Second Place Poster)   ***Western University, London, ON, CA   2014***

SEMINARS AND PRESENTATIONS

Functional Interaction of Connexin 43 and 40 Hemichannels in Gap Junctions
*Western University, Physiology and Pharmacology, London, ON, Nov 7, 2013, **(Talk)***

Critical Residues Responsible For The Formation of Functional Homotypic and Heterotypic
Gap Junctions of Major Atrial Connexins
*Western University, Physiology and Pharmacology Research Day, London, ON, Nov 1, 2014, **(Poster Presentation)***

Crucial residues for docking and formation of functional Cx40/Cx43 heterotypic gap junction channel
*Western University, Cardiac Biology Journal Club, London, ON, Jan 22, 2015, **(Talk)***

Variants located on E1 and E2 improve the efficiency of heterotypic Gap Junction
*Western University, Connexin and pannexin Group, London, March 5, 2015 **(Talk)***

Crucial residues for docking and formation of functional Cx40/Cx43 heterotypic gap junction channels
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