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Gap Junctional Intercellular Communication: Role of Cx43 Phosphorylation by Tyrosine Kinases

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GAP JUNCTIONAL INTERCELLULAR COMMUNICATION: ROLE OF Cx43 PHOSPHORYLATION BY TYROSINE KINASES

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GAP JUNCTIONAL INTERCELLULAR COMMUNICATION: ROLE OF Cx43 PHOSPHORYLATION BY TYROSINE KINASES

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Abstract

Phosphorylation of Cx43 is a process that regulates various functionalities of the gap junction including assembly, stability, internalization, turnover, channel permeability and channel gating. Src has been shown to phosphorylate Cx43 at Y247, Y265, Y313 and regulate gap junctions. Intercellular communication in B and T cells is necessary to mediate adaptive immune response. However, there is little-to-no expression of Src in these lymphocytes. An *in vitro* kinase screen identified Bruton's tyrosine kinase (BTK) and Interleukin 2-inducible T-cell kinase (ITK) to phosphorylate Cx43. Mass spectrometry identified Tyr residues to be phosphorylated by BTK and ITK, and these residues are similar to the ones phosphorylated by Src. Overexpression of BTK or ITK in HEK293T cells caused increased phosphorylation of Y247 along with decreased gap junctional intercellular communication (GJIC), without changing the cellular localization of Cx43. Similar results were observed with experiments in B and T lymphocytes as well. Our study indicates BTK and ITK to play a role in Cx43 phosphorylation-mediated regulation of gap junctional intercellular communication.

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Every experience helps shape us into the person we are. I thank all the people at the University of Nebraska Medical Center who provided me with this unique and special experience that will consciously and sub-consciously guide me towards the person I become in the future.

Dedication

I dedicate this to my parents and family in India; to my late grandparents; to my friends in India, USA and all over the world, who have acted as my stress relievers at different moments in my life.

We come across people, acquaintances and strangers, whose contributions seem to be little or insignificant at first, but over time we realize the immense significance of their contributions. I want to thank all of you.

Last but not the least, I dedicate this to my greatest therapy – Kathak dance and all the people who have supported me as a dancer. Its presence in my life increases my motivation towards my other interests, including scientific research.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

Introduction

1. Gap junction overview

Intercellular communication forms the backbone of the cellular networking system in an organism. All physiological systems including cardiovascular, neuronal, digestive, metabolic among others, depend on cell-cell communication to sustain the required mechanisms of the body. Intercellular communication can be direct or indirect. Direct communication is involved in receptor mediated signaling networks, transfer of neurotransmitters, maintaining cell polarity etc. One major player of this type of communication is the Gap Junction.

Gap junctions are intercellular channels that allow direct cellular transmission of ions and small molecules [1]. Each channel is formed of two hemichannels or connexons where each cell contributes one connexon [Fig. 1] [2]. The narrow extracellular gap between two connexons is approximately 2 nm [1]. Each connexon is composed of six connexin proteins. There are 21 known human isoforms of connexins. Different connexin isoforms can oligomerize to form a connexon. This varied combination determines if the hemichannel is homomeric or heteromeric. If all six connexins in a connexon are the same, they form a homomeric hemichannel, whereas different connexins form a heteromeric one. If the connexin compositions of both the hemichannels are identical, the gap junction channel is called homotypic, otherwise it is called heterotypic [3]. This differential arrangement is significant in providing specificity to the functioning of the gap junction channel. It also increases the functional diversity of the channels. For example, Cx32 homomers are equally permeable to cAMP and cGMP, but, Cx32-Cx26 heteromers are selectively permeable to cGMP [4]. From a few to thousands of such gap junction channels form a gap junction plaque at the cell membranes of two communicating cells as revealed

by X-ray diffraction studies [5]. Gap junctions in some of these plaques are stable while others are more mobile and fluid. Fluid gap junctions can arrange and rearrange themselves within a plaque [6]. In mice, this instability has been shown to cause white matter abnormalities and delayed neural differentiation [6, 7].Gap junctions at the plaque are associated with interacting molecules and form a Nexus of molecules. The variation in composition of the Nexus controls various cellular signaling processes [7].

The ions and small molecules being transferred through the gap junction channels need to be less than 2 kDa [8]. Inorganic ions, amino acids, sugars, RNAs, vitamins, second messengers are examples of substances that can pass through a channel. Gap junctions regulate intracellular Na+ concentration in astrocytes [9]. Passage of glucose through gap junctions of rat islet cells regulates insulin secretion. Passage of cAMP through channels plays an important role in maintaining bone homeostasis in osteoblasts [10]. Gene expression in neighboring cells can be regulated by miRNA transfer through channels [11]. Passage of serotonin trough channels promotes pulmonary arterial smooth muscle cell differentiation [12]. Electrical communication across gap junctions synchronize contractions in cardiac myocytes [13].

Besides connexins, gap junctions are also formed of innexins. Non-chordates have channels made of innexins. The early discovery of innexin was made in *Drosophila* where it showed to serve a similar purpose as that to connexins [14]. However, they have very little homology to connexins [15]. Despite lack in homology, the topological and functional attributes of the innexin and connexin based gap junctional channels are highly similar [16]. Connexins are said to have evolved from innexins after the latter started to lose their diversity [17]. Connexin proteins are most conventionally named after their molecular

weights. For example, Cx43 is a 43 kDa protein. Secondarily, they are also named according to sequence similarity, length of CL (cytoplasmic loop) domain and evolutionary homology [18]. Based on this method, connexins are divided into three major (α, β, γ) and two minor (δ or ε) [18]. The names begin with Gj (Gap junction), followed by the order of discovery of the specific family member [18]. For example, the Cx43 gene is call gja1 because this was the first discovered connexin of the α subtype. The protein is called GJA1. Connexins constitute the gap junction of all living chordates except lancelets [19]. Connexins are ubiquitously expressed in all cell types except in red blood cells and skeletal muscles [20].

2. Connexin structure

Connexins are four-pass transmembrane (TM1-4) proteins that have a cytoplasmic loop (CL), two extracellular loops (EL1 and EL2), one amino-terminus (NT) and one carboxyl terminus (CT). EL1 and EL2 have three disulfide bonds between three pairs of cysteine residues [21-26]. These are important for proper folding and docking of connexins and hemichannels. The TM and the EL are more homologous among connexin isomers. The homology of the cytoplasmic domains is much less and the greatest divergence is found in the CT domain. Conservation of amino acids of NT domain among connexins is very high [27]. The length of the NT domain is similar among connexin isomers. It is usually 22-23 residues in length [28], but there is considerable difference in the secondary structure. Nuclear Magnetic Resonance (NMR) technique has been extensively used to study structure of connexin domains. NMR has solved the structure of many NT domains. Cx32NT was seen to be disordered [29], whereas Cx26NT was seen to be α-helical in nature and a crucial bend was also noticed at residues 11 and 12 [30-32]. This bend causes

the first few residues to be directed more towards the pore [32]. Apart from NMR, crystallographic studies have also been significant in studying connexin structure [33]. With this technique it was observed that the NT domain was lining the vestibule of the pore. This causes changes in ion selectivity and voltage gating [33]. In fact, the NT half of the connexin has been shown to be the most critical domain to regulate voltage gating [33]. Crystallography was also used to study the open pore structure of Cx26. It was seen that interactions between the NT and the TMs are important for holding the channel in the open state. This study also indicated the formation of hydrogen bonds between D2 and T5 of promoters present at the narrowest point of the pore and were adjacent to each other [33].

The length of the CL domain varies among connexin isomers. There are connexins with small CL (30-35 residues), medium CL (50-55) and large CL (80-105) [34]. NMR and Circular Dichroism (CD) studies have shown that the CL is highly disordered [33] and also mostly exists in the α-helical form [35]. Two small α-helical regions were found in the CL domain [35]. The CL of Cx32, Cx36, Cx43 showed an increased tendency to form α-helical structures after addition of trifluoroethanol (TFE), which acts as a stabilizer of secondary structures [36]. These regions play roles in channel gating modulated by intracellular pH levels and binding of calmodulin [36]. A study showed that Calmodulin can interact with a Cx43CL peptide comprised of residues K136 – S158. This interaction was associated with increased α-helical contents in the Cx43CL [37]. Because of this disordered nature of the CL, it has been difficult to execute crystallographic studies to determine its structure. Even so, studies that included truncation of residues in the CL (residues 130-134 or 139-143), have shown the importance of the CL in connexin trafficking, channel formation and electrical coupling [38, 39].

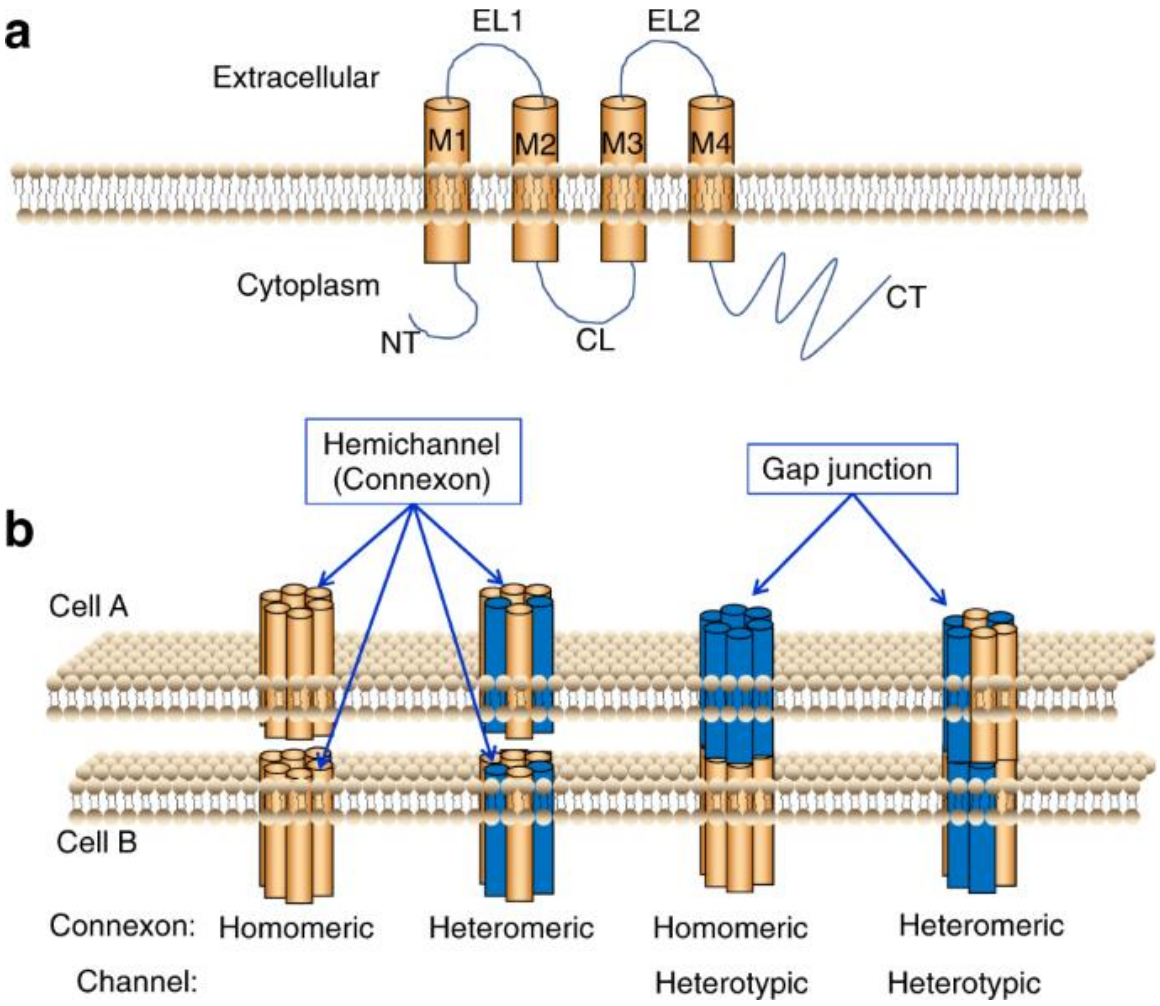


Figure 1: Gap junction and connexin structure

Model of a connexin and Gap Junction channel. **a**. Structure of a connexin. NT: N-terminus, CL: cytoplasmic loop, CT: C-terminus, EL1 and EL2: Extracellular loops 1 and 2, M1-M4: Transmembrane domains1-4, **b**. Possibility of connexin arrangement in a gap junction channel. Homomeric connexons: formed by one connexin type, Heteromeric connexons: composed of different connexin types, Homotypic channels: channels formed of 2 connexons with the same connexin types, Heterotypic channels: channels formed of 2 connexons with different connexin composition. Figure is adapted from [40].

3. Connexin related diseases and mutations

The ELs contain three pairs of Cys residues located at identical positions. These Cys residues form disulfide bonds which are necessary for proper folding of connexins and their proper docking. Mutation of any one of these cysteine residues to serine resulted in a loss of channel function in all the connexins tested [41]. The CL has many functions including proper electrical coupling and metabolic transfer [41, 42]. Deletion of amino acids 130-136 in the CL region led to blockage of electrical coupling and transfer of neurobiotin and lucifer yellow (these are dyes used to study Gap Junctional Intercellular Communication or GJIC) [42]. Mutation in the N-terminal of Cx43 was shown to form non-functional gap junction plaques [43]. Mutations in the N-terminal of Cx36 have pronounced negative effect on Mg2+ sensitivity of the gap junctional channels [44]. The CT of the connexins have various binding partners including kinases and cytoskeletal elements. These associations regulate several important structural and functional mechanisms. This will be discussed later.

Mutations in connexins have also been linked to various diseases in many organs and cell types. Cx32 mutations are known to cause X-linked Charcot Marie Tooth disease [45]. Cx26 mutations cause non-syndromic hearing loss [46]. Null mice homozygous for Cx26 die at the embryonic stage [46]. Cx26 mutations also cause Vohwinkel Syndrome, which affects the skin causing thick honeycomb like calluses on the palms and soles of feet of children [47]. Mutations in Cx46 and Cx50 in mice lead to cataract formation as they are required for proper lens development in humans and mice [48, 49]. In the embryonic stage, expression of Cx45 is the strongest. It is the only cardiac connexin known to be activated in the embryo during the stage of first contractions of the embryonic heart. Mice

deficient in Cx45 die at the prenatal stage [50]. Cx40 deletion causes atrial fibrillation [51]. Cx46 and Cx47 mutations cause Pelizaeus-Merzbacher-like disease, which is a rare, progressive disease of the Central Nervous System (CNS) that causes disorders of coordination, motor ability and cognitive functions [52, 53]. Cx30 mutation causes Clouston Syndrome, which causes abnormalities in the skin, hair and nails [54]. Of all the connexins, Cx43 is the most widely studied one. It is also the most ubiquitously expressed connexin. Various studies have shown adverse effects of Cx43 mutation or deletion. Cx43 knockout mice die at the neonatal stage [55]. Mutations of Cx43 cause Oculodentodigital Dysplasia [56]. It is a rare genetic disorder which is characterized by various symptoms including premature loss of teeth and microcephaly. Expression of gap junctional and mitochondrial Cx43 in heart is altered in hypertension, hypertrophy, ischemia-reperfusion injury, hypercholesterolemia and many other heart diseases [57-60]. Electrical coupling of the heart is disturbed due to reduced Cx43 causing ventricular arrhythmias [61]. Absence of Cx43 causes reduction in retinal size and depolarization of retinal progenitor cells [62]. Integrity of the myelin in the CNS is hampered due to lack of proper Cx43 function. This leads to impaired electrical transmission in the nervous system and impaired intercellular communication between oligodendrocytes and astrocytes [63]. Cx43 mutant mice also have defects in the auditory system and cause many skin diseases [64].

4. Connexin life cycle

The life cycle of connexins is similar among the different isoforms. The processes involved in the life cycle include transcription, translation, post-translational modifications, trafficking, assembly and finally turnover and recycling.

4.1 Biosynthesis

The life cycle of connexin begins with the transcription of the connexin genes. Many transcription factors regulate the gene activity and thus subsequently the expression of connexins. A basal transcription factor called Specificity Protein 1 (Sp1), binds to GC box sequences in promoter regions and is known to favor the initiation of transcription of many connexin genes [65]. Another important player is the Activator protein 1 (Ap1) transcription factor which has proteins from the c-Fos, c-Jun families [66-68]. The Activating Transcription Factor (ATF) and J domain containing protein (JDP) families promote positive regulation [67, 68]. The activation of the Wnt pathway causes the formation of nuclear β catenin/T-cell factor (TCF) complexes which act as transcription factors by binding to specific motifs in the promoter region of some connexin genes [69]. Epigenetic mechanisms such as histone acetylation and deacetylation also regulate connexin gene transcription. miRNAs and RNA binding proteins are involved in post-transcriptional regulation of connexins [70, 71]. For example, miR-1 downregulates Cx43 expression and Human Antigen R(HuR) stabilizes Cx43 mRNA [70]. Translation of connexins can be regulated by the regulation of internal ribosome entry sites (IRES) present on the connexin mRNAs [72, 73]. Post-translation regulation includes modifications such as Ser, Thr, Tyr phosphorylation, S-nitrosylation, sumoylation, ubiquitinatuon, methylation. Connexins are inserted into the ER. Oligomerization of connexins into connexons occurs in either trans-Golgi network (TGN), or ER-Golgi intermediate compartment (ERGIC), for example, oligomerization of Cx26 and Cx32 occurs in the ER membranes [74, 75]. Cx43 and Cx46 oligomerize in the TGN [76, 77]. This is followed by trafficking of connexons to the plasma membrane (PM).

4.2 Trafficking and assembly of gap junctions

There exist a few different possibilities of recruiting newly formed connexons to the plasma membrane. Fusion of the transport vesicles that contain connexons can occur with the plasma membrane at the outer margin of the plaques, or with the plasma membranes in the plaques or with non-junctional plasma membranes which is followed by the connexons drifting across the membrane to dock and form gap junction channels [78]. Studies conducted by tagging the CT of connexins with Green Fluorescent Proteins (GFP) and Cyan Fluorescent Proteins (CFP) have shed some light on the trafficking of connexons. These studies have indicated that microtubules play major roles in trafficking of connexons [79]. Phosphorylation of the connexins is critical in regulating transport to the PM. Ezrin protein (binds to actin), 14-3-3 protein and cAMP dependent protein kinase A (PKA) are known to be involved in the recruitment of connexin-containing transport vesicles into the plasma membrane [80]. It has been shown that Zonula Occludens-1 (ZO-1) protein regulates the rate of connexon aggregation into gap junctions. ZO-1 binds to the CT of Cx43. Inhibition of this interaction has been shown to increase the aggregation of gap junction. The domain adjacent to gap junctions is called perinexus [81]. Inhibition of ZO-1/Cx43 showed reduced perinexal/connexon interaction and increased the ratio of docked connexons in the gap junction to undocked connexons in the plasma membrane [81]. Phosphorylation by PKA has been shown to increase stability of gap junction plaques and increases the assembly of new gap junctions. Actin cytoskeletal elements and associated proteins also serve to stabilize gap junctions at the PM. A membrane cytoskeletal protein called α-spectrin was also showed to co-precipitate with gap junctional complexes by interaction with ZO-1. Another cytoskeletal element called Drebrin (developmentally

regulated brain protein), which binds to actin, regulates cellular polarity and stabilization of Cx43 gap junctions by binding to the CT domain [82]. siRNA knockdown of Drebrin caused inhibition of cell coupling, leading to internalization and degradation of Cx43 [83]. Adherens junction proteins such as cadherins interact with connexins to mediate proper gap junction assembly. N-cadherin has been shown to co-localize and co-interact with Cx43 at the cell-cell contact region [84]. N-cadherin-Cx43 association requires Rac 1 and RhoA dependent signaling and leads to delivery of connexons and their insertions into the PM [85]. GJIC was also showed to be increased following upregulation of N-cadherin-Cx43 association [86]. The α-catenin and β-catenin proteins also interact with CT of connexins and regulate trafficking of connexons and assembly of gap junctions [87]. Some other connexin interacting partners include calcium/calmodulin-dependent kinase II (CaMKII), which phosphorylates certain connexins to enhance gap junctional coupling [88]. Cholesterol interaction with gap junctions has also been studied to some extent [89]. Newly formed gap junctions are found to be cholesterol rich [90]. In adult chicken lens, it was found that during fiber cell maturation, there is significant gap junction remodeling occurring. New cholesterol rich gap junctions with loosely packed connexons were found to be transformed into cholesterol free ones with tighter connexons [90].

4.3 Internalization and turnover

A connexon may get internalized into its cell or a whole gap junction composed of both the connexons may get internalized into one of the cells. Both these methods have been noticed in studies [91]. In the latter method, the gap junction complex is called annular junction or connexosome. A double membrane vesicle forms around the connexosome and the vesicle then gets internalized. Here, the cell into which the connexosome is internalized

is called the acceptor cell, whereas the other is the donor cell [92]. Immunolabeling of annular junctions have shown that these structures do contain largely connexins [91, 92]. There are both clathrin-dependent as well as clathrin-independent methods [92, 93]. The process requires association of adapter proteins such as AP-2, Dab 2 and dynamin [93]. A special type of lipid raft called caveolae can also be involved in internalization. Cx43 and Cx26 colocalize and coimmunoprecipitate with these lipid rafts indicating an association of lipid rafts/connexins [94]. Recently it has been shown that concurrent occurrence of new connexons getting integrated into the gap junction plaque happens with the partial internalization of the central connexons [95]. Thus, there are quite a few mechanisms that could mediate internalization.

Unlike many other membrane proteins, connexins have unusually short half-lives, usually 1-5 hours [96]. This varies from one cell to another, but the range has been shown to be consistent among various connexins studied such as Cx26, Cx32, Cx37, Cx43, Cx45. Differential binding to partners and differential phosphorylation are involved in the variation of half-lives [96, 97]. The short life cycle has been well studied in both cultured cells and 3D (3-Dimensional) conditions [97, 98]. Thus, connexins are constantly synthesized and degraded. One explanation for such an occurrence is that it is necessary for the connexins to quickly respond to certain physiological conditions and quickly regulate the gap junctional coupling. It has been studied in the myometrium that just prior to labor, there is rapid (almost 5- fold) increase in the level of total gap junctions probably promoted by high levels of steroid hormones. This is followed by rapid significant decrease in the gap junction levels just after labor, when the GJIC returns to the steady-state level

[99]. The internalization process is followed by the final step of the life cycle which is degradation.

Both lysosomal and proteasomal mechanisms are involved in degradation. In perfused rat hearts, it has been shown that inhibition of lysosomal and proteasomal degradation pathways leads to increase in Cx43 expression [100]. In one study, inhibiting proteasomal pathway led to accumulation of non-phosphorylated Cx43, whereas another study showed that Cx43 phosphorylation along with GJIC was increased following proteasomal inhibition [100, 101]. Even though these results are contrasting, they show that phosphorylation plays a critical role in connexin degradation. A study also showed involvement of proteasomal pathway in the degradation of Cx32 and Cx43 occurring in the ER. Most of the degradation of these two connexins are happening via ERAD. Gap junction channel formation and function increased after inhibition of ERAD [102, 103]. It is thought that regulation of ZO-1, which binds to Cx43 CT, by ERAD may be responsible for regulating gap junctional stability. Electron microscopy detected the presence of connexosomes inside lysosomes [104]. The Cx43 half-life has been shown to increase after inhibition of lysosomes [104]. Inhibition of lysosomes almost eliminated the phosphorylation of Y247 and Y265, while reducing that of S368 and S373, but increased S279/282 phosphorylation. On the other hand, inhibition of proteasome increased the phosphorylation levels of Y247, Y265, S279/282, S365, S373. This indicates that phosphorylation regulates gap junction disassembly and has differential effects on cellular functioning [105].

Another major process of connexin degradation is ubiquitination. It has been seen that ubiquitination occurs after specific phosphorylation events. Phosphorylation of certain

Ser residues increases the binding affinity of an E3 ubiquitin ligase called neuronal expressed developmentally downregulated protein 4 (NEDD4) to Cx43 [106, 107]. This ubiquitination process involves recruitment of Adapter protein2 (AP2) and clathrin to mediate internalization and transport of connexins to the lysosome. Eps 15 interacts with ubiquitinated Cx43 leading to degradation of Cx43 via endocytic pathway [108].

Autophagy is another pathway detected in degradation of connexins. During autophagy, the proteins destined for autophagy get entrapped in the autophagosome. Different connexins such as Cx26, Cx32, Cx43 were found to be trapped in their WT forms inside the autophagosomes [109]. Also, colocalization of the autophagy related protein, LC3 with connexosomes has been detected. Intracellular connexins were found to colocalize with p62 protein, which serves as a cargo receptor for autophagosomal degradation [109]. By expressing fluorescent tagged Cx43 in HeLa cells and analyzing the ultrastructure of Cx43, it was showed that gap junctions are degraded by autophagy [95].

5. Channel gating

A gap junction channel or a hemichannel can exist in two states: open and close. This phenomenon is called gating of channels. Channel gating is a significant method of regulation of ions and metabolite passage through them. Interfacial energy maps have been used to determine the hydrophilic and the hydrophobic surfaces of the channel, both on the Cx43 ELs and the pore mouth [110]. While in the closed state, the ELs are tightly packed. But, in the open state they are at least partly unfolded (the pore is larger with larger outer diameter). The open and closed diameters of Cx43 channels are 2.5 nm and 1.8 nm respectively. In case of Cx26 channels they are 1.5 nm and 0.6 nm respectively [111]. There are various factors that can affect the state of the channel. Voltage gating, pH gating

and Ca^{2+} gating are three such factors which can determine whether the channels are open or close. Thus, a single channel keeps switching between open and closed states.

5.1 Voltage gating

Gap junction proteins contain a range of charged amino acid sequences that act as a voltage sensor [112]. These residues constituting the voltage sensor are present on the N-terminus and at the border of TM1-E1 [112]. There are two types of voltage that determine the voltage gating: transjunctional (Vj) and transmembrane (Vm), the former being the more common. Channels face loss of function due to shifts in voltage-dependency, as the channels are closed at voltages where they should be open. It has been seen that undocked hemichannels have low open probability [110]. Despite that, they play major roles in maintaining physiological functioning of the organism. In excitable cells, such as nerve cells, heart cells and smooth muscle cells, the regulation of undocked hemichannel can alter the membrane potential to modulate electrical signaling and excitability [110]. However, at the resting potential, closure of undocked hemichannels is strongly favored [110].

Studies showed that there are two types of Vj gating: slow gating and fast gating [113]. In slow gating, the gating transition between open or closed state and a transient substate is slow. A series of transient substates exist between the open and closed state. In contrast, fast gating refers to the gating transition between the open state and a substate being faster [113]. In HeLa cells, stably expressing Cx43, slow gating corresponds to a mean transition time of 10ms, while fast gating transition occurs in about 2ms [114]. The intracellular structures of the connexin: the NT, the CL and the CT are all involved in voltage gating. The NT seems to play the most significant role. Mutation of certain amino

acids in the NT of Cx26 and Cx32 showed that this region is key for Vj gating [32, 112]. Truncation of Cx32 and Cx43 at key residues of the CT resulted in slow Vj gating, indicating a role for the CT domain in fast Vj gating [115]. It is predicted that under normal conditions, the CT is away from the pore. After the occurrence of a stimulus, the CT moves closer to the channel, interacts with a CL and ends up blocking the channel [116].

The less common Vm gating has been found in Cx43, Cx45, Cx57 [117-119]. The Vm gating dependency may vary among different cell types as it was seen that there was high Vm gating of Cx43 channels in oocyte as opposed to low Vm gating in HeLa cells [119, 120].

5.2 pH gating

Concentration of H+ ions is an important determinant for gating states of a channel. High acidity (pH 5-7) has been shown to close most gap junction channels [121, 122]. This indicates that the gating mechanism of channels is trying to protect the cells from adverse effects of higher acidity. At physiological pH, which is around 7.4, different connexin isoforms show different opening rates. For example, Cx43 channels mainly remain open at pH 7.4, but very few Cx57 channels remain open [123, 124]. The secondary structures of different CT domains vary at different pH levels. At lower pH (5.8), Cx43 CT is α-helical, whereas there is very little α-helix at higher pH (7.5) [125]. In other connexins too, higher acidity tends to increase the α-helical structures of soluble connexin CT domains. Cx46 hemichannels showed rapid closure as a result of low pH application [126]. The H+ binding site, also known as the pH sensor site, is located on the cytoplasmic side and is predicted to be near the opening of the pore [126]. Along with the CT domain, involvement of CL domain in pH gating has also been shown in *Xenopus* oocytes. pH sensitivity decreased

after truncating the Cx43 CT. Co-expressing the truncated Cx43 along with WT Cx43 CT restored the pH sensitivity [123]. Cx32 is less sensitive to pH compared to Cx43. When Cx32 was co-expressed with Cx43, pH sensitivity was increased [127]. This pH gating property of the channel has also led to pharmaceutical advances. There is a compound called cyclized heptapeptide (CyRP-71), which can bind to the Cx43CT to partially inhibit channel closure by increasing the acidity [128].

Some studies have established a link between Vj gating and pH gating. In Cx45 homotypic and Cx45/Cx43-enhanced GFP heterotypic channels, increase in pH increased the Vj gating dependency, thus increasing open probability whereas decrease in pH decreased Vj gating and led to channel closure [129]. Apart from gap junction channels, hemichannels are also pH dependent [130-132]. It has been observed that increase in intracellular acidity leads to long-term channel closure and increase in extracellular acidity also leads to channel closure, but it is more rapid, and the channel quickly reverts to its open state [126]. Also, under positive potential conditions, addition of weak membrane permeable acids extracellularly, prevented hemichannel openings. On the other hand, strong acids did not affect the opening of closed hemichannels, but rapidly closed the already open hemichannels [126]. pH gating plays a role in both normal and pathological conditions. Increased neural activity corresponded to a shift in intracellular pH (pHi) of 0.2-0.4 units and this was mediated through gap junction related neuronal signaling [133].

pH gating of channels does not depend on one mechanism. There have been many different mechanisms proposed to describe the effect of pH on channels including protonation of taurine residues, involvement of bivalent cations and direct protonation of connexin [134-136]. A study proposed that Cx26 gap junction channels and hemichannels

are affected by H+ ions through protonation of taurine residues, which causes inhibition of gap junction channels [134]. Some studies have found a link between pH and bivalent cations such as Mg2+ and Ca2+ in channel gating. A study suggested that the uncoupling effect produced by pHi can be attributed to an increase in Ca2+ levels [135]. Certain connexin residues can be protonated. Direct protonation of connexin residues can also affect pH mediated junctional gating [136]. Recent studies have shown that Cx36 gap junction channel activity is directly modulated by pHi. H18 residue of the NT domain of Cx36 is critical for the channel uncoupling due to alkalization [137].

5.3 Ca2+ gating

Among the bivalent cations known to affect gap junction channel gating, Ca2+ is one of the most highly studied. High intracellular levels of Ca2+ can inhibit gap junction communication. Two mechanisms have been proposed for Ca2+ related gating: one is direct binding of Ca2+ to connexin hemichannels and the other one being interaction via a modulator called calmodulin (CaM) [138, 139]. Direct interaction of Ca2+ with connexin causes closure of hemichannels [138]. When Ca2+ binds to negatively charged residues of the connexin, it disrupts the salt bridge between the hemichannels in the extracellular regions, resulting in a conformational change [138].

CaM can interact with the cytoplasmic domains of connexin in presence of Ca2+ [139]. Ca2+/CaM dependent gating has been studied in various connexin types such as Cx32, Cx36, Cx43, Cx44, Cx45, Cx50 [140-146]. This CaM-connexin interaction causes a conformational change on the connexins [144]. Biophysical studies have indicated direct binding of CaM to Cx43 CL mediated by Ca2+ ions. CaM binding has also shown to increase the α-helical content of the Cx43 CL [37, 147]. Two CaM binding sites have been

found in Cx32, one in the NT and one in the CT. Just like the CL, the CT also showed increased α-helical content after CaM binding [37, 148]. This pattern of increased α-helical content was also found in other connexins such as Cx44 and Cx50 [144, 146]. The conformational change caused as a result of CaM binding may be blocking the gap junction pore. Inhibiting CaM -connexin interaction caused the gap junction pore to revert from closed to open state [149].

5.4 Mg2+ gating

Recently, it has been shown that there is direct interaction between Mg2+ ions and gap junction channels [44, 150]. Sensitivity of Cx36 gap junction to high levels of Mg2+ has been shown to be determined by aspartate residues [151]. It has been shown that gap junction channel gating during acidification is dependent on Mg2+ ions, supporting the existence of interdependence of factors that affect channel gating [152]. It is seen that acidic pHi causes decrease in junctional conductance (jc) only at low Mg2+ levels [137]. NT of connexins play a possible role in Mg2+ related gating just like in H+ related gating. There are regions of various negatively charged residues that can bind to bivalent cations [152]. Histidine residues can also get protonated at physiological pH and affect the gap junction gating [153]. The NT has negatively charged glutamates that form the vestibule of the gap junction channel, can be involved in binding cations to facilitate gap junction channel passage of ions. It has been seen that at high intracellular Mg2+, E8Q and E12Q mutations removed the stimulatory effect caused by acidification [153]. It is proposed that Mg2+ and H+ directly bind to glutamate E8 and E12 to regulate gap junction channel gating; however, more studies are required to elucidate the exact mechanism [153]. Besides

the above-mentioned factors, protein partner binding and phosphorylation of the CT domain play major roles in regulation of channel gating.

6. Connexin CT domain

Diversity of the connexin CT domain has been observed in size, amino acid sequence, protein partners for binding. The length of the CT can be short (as in 10 residues for Cx26) and very long (as in 310 residues for Cx62). The CT domain has been found to be distorted and dynamic in nature, thus cannot be subjected to crystallographic studies. These properties have restricted the studies to NMR and CD spectroscopy. The CT domains of many of the connexins was found to be primarily disordered by solution NMR and CD studies [35, 154]. These studies also revealed that these CT domains contain predominantly α-helical regions [154]. This α-helical secondary structure contributes to dimerization of the CT domains [35]. It also aids in pH dependent gating via the interaction of CT-CL domains [35]. Studying the Cx43 CT-TM tethered domain by NMR revealed that there are actually seven α-helical regions [155]. Two of these (H4 and H5) were revealed while expressing the Cx43 CT without tethering to the TM domain [155]. These two were however found to exist within longer α-helical domains when TM was tethered [155]. Besides Cx43, similar α-helical domains were also found in Cx45, Cx32, Cx36 [36]. Cx45 CT was seen to have the largest α-helix [156]. The presence of α-helical structures may help in bringing more order to the connexin structure, help in chemical gating and aid in protein partner binding [154][156].

As briefly mentioned before, post-translational modifications of the connexin CT domain play major roles in dictating the cellular functioning of the cell. Examples of these modifications are phosphorylation, ubiquitination, hydroxylation, S-nitrosylation,

palmitoylation, oxidation (for review, see [157]. Two best studied methods among these are phosphorylation and ubiquitination. Since phosphorylation mainly happens at a disordered region of the protein, its frequency is very high [158]. This disordered nature helps to form hydrogen bonds between kinase enzymes and their substrates, which would be hampered if the kinase binding regions were more ordered [158]. Phosphorylation causes changes in the charge of a molecule by adding two negative charges at physiological pH [159]. It also changes the hydrophobicity and brings more order to the protein structure, thus transforming them from the disordered to the ordered state [160, 161]. These changes have significance in channel gating, channel permeability, assembly, disassembly and internalization. The thermodynamic favorability of protein-protein binding also changes due to phosphorylation, thus regulating protein interactions [162]. Many phosphorylation sites on connexin CT domains have been found to be within protein binding sites [156, 163].

Various protein partners have been identified for the connexin CTs. Some promote intercellular communication, while others impede communication. Kinases, phosphatases and cytoskeletal element are the most well studied binding partners. Tubulin, Drebrin, ZO-1, c-Src, Casein Kinase (CK-1), Mitogen Activated Protein Kinase (MAPK),PKA, CaM are among such interacting partners [149, 164-168]. The disordered nature of connexin CT facilitates low affinity and high specificity of proteins for the CT, thus, making it more favorable for rapid signaling changes for required intercellular signaling [154, 169].

The connexin CT is capable of regulating cell growth in both the cytoplasm and nucleus [170-173]. Transfection of cardiomyocytes and HeLa cells with Cx43 CT showed that cell growth is inhibited due to the nuclear localization of the CT [174]. Cx43 CT is

15kDa, but in this study the size of the CT was found to be 20kDa. The increase in size suggests that a moiety may have been added through post-translational modification [174]. In another study where Cx43 CT was downregulated in human breast cancer samples, there was decrease in p53, whose wild type form has cancer protecting properties. This indicates an effect of nuclear localization of CT on gene expression [173]. This effect may not be due to direct binding of CT to the DNA as no DNA-binding motif has been identified. The interaction may be occurring between the CT and transcription factors or other transcription regulators to regulate gene expression [173].

6.1 Post-translational modifications (PTMs)

6.1.1 Connexin ubiquitination

Protein degradation and turnover are required to maintain the balance between the increase and decrease in cellular signaling processes. Ubiquitination is a method that accomplishes this with the help of a 76-residue polypeptide called ubiquitin, which is ubiquitously expressed [157]. This process requires binding of ubiquitin to the protein desired to be degraded. Firstly, ubiquitin binds to the ubiquitin activating enzyme (E1 family of ubiquitin ligases). This is followed by its transfer to an ubiquitin carrier (E2 family of ubiquitin ligases). The E3 family of ubiquitin ligases then comes into play. It helps in transfer of the ubiquitin to the substrate and requires substrate specificity. The ubiquitin is transferred covalently from lysine 76 of ubiquitin to lysine residues on the protein of interest via an isopeptide bond. This may but not necessarily be followed by further ubiquitination via binding to ubiquitin linkages. Any of the Lys residues on the ubiquitin molecule can contain these linkages [175]. The K48 polyubiquitination chains prime the protein target for proteasomal degradation [175].

Ubiquitination is a process that works with ERAD (Endoplasmic Reticulum Associated Degradation) for connexins as well as other proteins. About 40 % of newly synthesized Cx32 and Cx43 undergo ERAD. This is correlated with high levels of cellular stress. A Cx32 mutation conferring CMTX caused almost all the Cx32 to be primed for ERAD [176]. Cellular stress causes reduction in degree of polyubiquitination causing misfolded Cx32 to accumulate in the ER. This misfolded Cx32 oligomerizes with the remaining WT Cx32 in the ER leading to improperly functioning Cx32 connexons causing diseases [177]. The connexins that are already incorporated into the gap junction plaque are unbiquitinated by NEDD 4, which then causes endocytosis of the connexins, followed by internalization and targets them for lysosomal degradation [178].

Since connexins themselves play roles in many physiological conditions, their ubiquitination also has a wide range of physiological implications as their turnover affects cellular signaling processes and cell-cell communication [179]. The role of connexin ubiquitination in the heart, lens and nervous system have been extensively studied in recent years [180-182]. Coupling of cardiomyocytes by gap junctions is necessary for maintaining normal heart rhythm. In neonatal ventricular rat cardiomyocytes, connexins bind to NEDD 4 and are subjected to ubiquitination [183]. Studies found a role for the E3 ubiquitin ligase WWP1, a NEDD 4 family member, in regulating degradation and gap junction size in cardiomyocytes. The authors established that overexpressing WWP1 globally or in a catdiomyocyte-specific manner caused lethal ventricular arrhythmias [180]. Studies have found links between ubiquitin-proteasomal system, gap junction and lens clarity. A study showed that hampering the ubiquitin/connexin axis is the lens resulted in increased Ca2+ levels, which resulted in cataract [184]. Overexpression of LNX1, an E3 ubiquitin ligase

in the central nervous system, led to loss of function of Cx36 gap junction, resulting in disrupted electrical synapses and signaling [182].

6.1.2 Connexin SUMOylation

There is a small family of proteins called small ubiquitin like modifier (SUMO) proteins, which have structural and functional similarities to ubiquitins. In humans, there are three members of SUMO protein family (SUMO1,2,3) [185]. Similar to ubiquitination, SUMOylation includes attachment of SUMO to the lysine residues of the target protein [157]. Sentrin specific proteases (SENPs) cleave a C-terminal peptide from SUMO and reveal a diglycine motif. This is followed by binding of SUMO to an E1 enzyme. It is then transferred to E2, which is a conjugating enzyme, that directly recognizes substrates with a consensus sumoylation motif. Then, the SUMO may or may not be transferred to E3 ligase enzymes that attaches SUMO to target proteins [157, 186]. Unlike ubiquitination, where E3 ligase is necessary, in SUMOylation it is optional. Also, unlike ubiquitination, SUMOylation does not destine the target protein for degradation. Instead, it is involved in regulating various cellular processes including transcription regulation, stress response protein targeting and stability, cell cycle progression and apoptosis [186, 187].

In the first study describing SUMOylation of connexins, HeLa cells were transfected with all the three families of SUMO [188]. This caused an increase in connexin expression and gap junction formation. SUMO 2 and SUMO 3 resulted in doubling of Cx43 expression and SUMO 3 also increased intercellular gap junctional communication [188]. Of all the connexins, Cx43 is the most well studied when it comes to SUMOylation. Mutational scanning of Cx43 showed that SUMO1-3 attached SUMO groups to K144 of the intracellular loop and to K237 in the CT of Cx43. Both these lysines are known to be

conserved among connexins. K144 has been shown to be conserved in 8 of the 21 connexin isoforms [188]. This suggested regulation of other connexins by SUMOylation.

Recent studies have found SUMOylation to have implications in cancer and other pathological conditions. Increasing the SUMOylation of Cx43 in liver cancer stem cells showed to cause an increase in gap junctional communication [189]. Ub9 is one of the SUMO conjugating enzymes. In osteosarcoma cells, silencing Ub9, causes decoupling of SUMO from Cx43, leading to increase in levels of free Cx43 [190]. This is important for reconstructing GJIC and recovering cellular functions [190].

6.1.3 Connexin S-nitrosylation

This post-translational modification involves reversible, covalent addition of NO to the thiol side chain of Cys residue [191, 192]. Multiple nitrosylases and denitrosylases act together to regulate the degree of nitrosylation [193]. Although many proteins have multiple Cys residue, it is seen that only very few thiol groups undergo nitrosylation in a single protein [191-193]. Nitrosylation directly affects cellular functions along with indirectly affecting functions through regulation of other post-translational modifications such as ubiquitination and phosphorylation [193].

Both hemichannels and gap junctions are affected by levels of NO [194]. the myoendothelial junctions (MEJs) of the vascular walls coordinate the activity of Endothelial Cells (EC) and Vascular Smooth Muscle Cells (VSMC) [195]. It was found that the MEJs have a high amount of eNOS, coexpressing with gap junction plaques [195]. Cx43 is constitutively nitrosylated in MEJs. Addition of phenylephrine induces denitrosylation and causes reduction in the movement of inositol triphosphate from VSMC

to EC via the MEJ gap junctions [196]. This decreased gap junction permeability was related to decreased nitrosylation of Cys 271 in the CT [196]. NO donors have been shown to decrease the electrical coupling of Cx37 gap junctions and the permeability of ECs towards small molecules [197, 198]. Increased NO production is also associated with ischemia and hypoxia [194, 199]. This was also correlated with an increase in Cx43 and Cx46 hemichannel activity [194]. This increase in hemichannel permeability may cause loss of intracellular ions and small molecules leading to cell death [194]. Recently, S-nitrosylation of Cx43 in astrocytes has been associated with oxidative stress, thus indicating an effect of connexin nitrosylation on the nervous system [194, 200].

6.1.4 Methylation and acetylation

Major function of methylation and acetylation is in epigenetic regulation. But there have been recent studies on their role in connexin related cellular functioning [201]. Mass spectrometry (MS) data showed that Cx26 was both methylated and acetylated. Mutation in a methylation site on Cx26 (R75W) showed to be associated with deafness, indicating a role of connexin methylation in pathophysiological roles [157, 201]. This mutation also reduced electrical and metabolic coupling. Mutation of methylation site on Cx30 has also caused disruption of inner ear functioning [202, 203].

Like methylation, acetylation of Cx26 also plays important roles in biological functioning [201]. Mutation of acetylation sites in Cx26 including K15 and K102 causes diseases [201]. Acetylation has also been seen in bovine Cx49 [204]. In mdx mice, a model for muscular dystrophy, interference with Cx43 acetylation-deacetylation caused its levels to decrease in the lateral membrane of epithelial cells (delateralization) [205]. Also, acetyl-

mimetic mutations in Cx43 caused an increase in intracellular localization of the connexin [205].

6.1.5 Glutamate γ-carboxylation

This post translational modification includes a vitamin K dependent conversion of glutamic acid to γ-carboxyglutamic acid [206]. Mass spectrometry results showed Glu42 and Glu47 of the EL and Glu114 of the CL of Cx26 to be γ-carboxylated [201]. This irreversible modification generates a high affinity Ca2+ binding site, thus indicating that γ-carboxylation of Cx26 may be related to Ca2+ sensitivity of this connexin. Furthermore, mutations of Glu42 and Glu47 causes Cx26-mediated deafness [201, 207], indicating the importance of γ-carboxylation in connexin function.

7. Phosphorylation

Phosphorylation has been detected in most of the connexin isoforms (including Cx32,37,43,45,46,50) and on all the intracellular connexin domains [168, 197, 208-217]. Although CT domain is the major site of phosphorylation, Cx26 NT, Cx36 CL and Cx56 CL phosphorylation have also been shown to exist [201, 216, 218, 219]. Since, Cx26 CT phosphorylation has not been detected yet, it is suggested that CT phosphorylation is not essential for channel formation [220]. Thus, it is suggested that CT-phosphorylation acts to regulate aspects of connexin functioning other that channel formation. CT-phosphorylation has been associated with all stages of the connexin life cycle and channel selectivity and conductance states [208, 221].

Connexin phosphorylation is the most widely studied PTM and to date, many different phosphorylation sites of connexins have been characterized. Cx43 itself has more

than 20 sites and various phosphorylating partners (for review, see [208, 222]). Ser, Thr and Tyr phosphorylation of connexins have major implications in biological functions [208]. Phosphorylation has been shown to regulate connexin in all stages of the life cycle including trafficking, assembly, disassembly, internalization, degradation, electrical gating, metabolic coupling. Most of the phosphorylation on connexins has been seen by Ser/Thr kinases [208]. They include PKC, MAPK, p34 (cdc2)/cyclin B kinase, PKA, CK1, p34cdc2, PKG, CaMKII, Akt [209, 223]. The first few identified Tyr kinases to phosphorylate the CT domain are c-Src, Fps, EGFR [224-226]. Among these, Src is the most widely studied Tyr kinase phosphorylating the CT. I will describe the Src phosphorylation of Cx43 CT in details later, as it forms the premise of the hypothesis for my work.

7.1. Methods to study connexin phosphorylation

In the initial phases of studying phosphorylation, ^{32}P or orthophosphate radiolabeling technique was used to label the proteins. Due to lack of phosphorous atoms in the standard amino acids, presence of ^{32}P indicates phosphorylation. The specificity of the antibodies for immunoprecipitation needs to be very high, but highly specific monoclonal antibodies were absent. This was a disadvantage of the method. After immunoprecipitating the connexin proteins from the radiolabeled cells, SDS-PAGE was used to detect phosphorylation using autoradiography [227]. Another disadvantage was that this method only identified phosphoproteins but could not identify the number of phosphorylated residues or their identity. Another method called phosphopeptide analysis has been used to approximately identify the number of phosphorylated residues and to detect changes in the phosphorylation of connexin comparing basal levels with levels under

stimuli by developing a 'phosphorylation fingerprint'. This method was different than the previous one such that here, there was a trypsin digestion done of the immunoprecipitated radiolabeled proteins, followed by a 2D electrophoresis on thin layer cellulose [227]. One disadvantage is that the trypsin digestion can be partial. The main classical method to identify phosphorylation is to use phosphor specific amino analysis (pSer, pThr or pTyr) and to compare their relative percentages. The method of fragmentation and the size of fragments formed differ among the last two methods [227]. In phosphopeptide analysis, trypsin digestion produces peptides of variable sizes. In phosphoamino analysis, free amino acids are generated by acid hydrolysis. Acid hydrolysis is followed by resolution of the sample with 2D electrophoresis and is then detected by autoradiography. The conditions used for the 2D electrophoresis also vary between these two methods [227]. Although these classical methods provided insight to connexin phosphorylation, they all needed the amount of starting protein to be much greater than the amounts needed to be experimentally feasible. They also lacked the ability to identify specific residues that are phosphorylated and to connect those sites to specific functions. Later, site directed mutagenesis and Edman degradation (as of Cx43 pS368 and pS255/279/282) were used to identify specific sites of phosphorylation of connexin [228-230]. A disadvantage of the Edman degradation method is the limitation of read length. An advantage of the Edman method includes the capability of using recombinant CxCT domains for *in vitro* phosphorylation so that there can be high levels of protein to start with. Another advantage was bypassing the use of radiolabeled substances.

When running connexin proteins on Western blot, different migratory patterns with differing electrophoretic mobility of bands have been observed (in Cx32, Cx40, Cx43,

Cx45, Cx46, Cx50) [215, 231-235]. The slower migrating bands have been shown to collapse or disappear upon phosphatase treatment [220]. The migratory pattern of Cx32 consisted of a doublet of bands which reacted to general pTyr and general pSer antibodies. These bands collapsed into a single band and reacted only to antibodies against Cx32 [231]. When Cx40 was extracted from arterial tissue, similar doublet was observed. The same was observed for Cx45 when extracted from HeLa cells [233, 234]. When extracted from mouse or mink seminiferous tubules, Cx50 doublet was seen, whose slower migrating band collapsed due to phosphatase [234]. In a study, *in vitro* phosphorylation of GST-Cx50CT by PKA showed a doublet. However, this was not reproduced in cell culture studies, where PKA activation in cells showed only a single band [215]. Doublet bands were also seen in Cx46 blots, both of which reduced in intensity upon phosphatase treatment. This suggested that non-phosphorylated Cx46 undergoes rapid degradation [234]. On the other hand, Cx43 shows more than two bands. The number of bands can be 3-5 or sometimes even more [209, 236]. The experimental and phosphorylation conditions may play a role in determining the number of bands. These bands start from the P0 (42kDa), to slower migrating (P1, P2 and above) [237]. The P2 form has been shown to be present in Normal Rat Kidney (NRK) cells with normal communication. These were primarily present in a detergent insoluble gap junction fraction which corresponds to the connexins present in the gap junction plaque [238]. In a study using cell lines lacking intercellular communication (S180 and L929), this P2 form was undetected along with insoluble Cx43 [238]. Thus, a link between the electrophoretic migratory isoforms and physiological functions was indicated. Later, link between the migratory forms and specificity of phosphorylating kinases were shown to exist. The P2 isoform was linked with phosphorylation of Cx43 by

Casein Kinase 1(CK1) and it was shown to require S325, S328 and S330 residues [165]. Phosphomimetic mutants of some known kinase phosphorylation sites of Cx43CT and TM4/Cx43CT and biophysical studies were used to study the identity of the migratory forms. It was seen that the soluble and the TM-tethered mutant Cx43CT both had slower electrophoretic mobility as seen in the P2 form [165, 239]. Similar phosphomimetic mutant studies were used for Cx43CT sites for PKA, PKC, MAPK, CDK1 and Src [239]. The P1 form was noticed only in PKA and MAPK. For kinases that phosphorylate multiple residues of Cx43CT, substitution of just one of the amino acids did not have an effect on electrophoretic mobility shift [239]. Disrupting the binding ratio of SDS to protein from the negatively charged phosphate has been suggested to cause the slower migration of phosphorylated proteins compared to non-phosphorylated ones. Phosphorylation sites adjacent to proline residues cause conformational changes or kinks that also affect the electrophoretic migration [240, 241].

Physiological relevance of connexin phosphorylation can only be understood with studies that include amino acid residue specific identification methods. Two current methods used for this purpose are use of phosphospecific residue antibodies and Mass spectrometry analysis. MS analysis helps us to detect site specific phosphorylation even at femtomolar range [242]. This is also a very quick process of analysis. Currently, there are facilities with tandem MS/MS instruments, which are now highly preferred [243]. Like other methods, MS also poses some challenges. Since, phosphorylated proteins are more abundant than non-phosphorylated ones, the phosphopeptides usually produce less signal comparatively. It can be difficult to analyze the properties of collision induced decay phosphopeptides using MS [243]. A method to mitigate this drawback has been to enrich

phosphopeptides. On way to do this is to pull down the phosphopeptides beforehand by using antibodies against general phospho antibodies [244]. Another method of enrichment is the immobilized metal affinity chromatography. In this case, many immobilized metal ions in the solid phase can separate the proteins to variable degree depending on affinity towards the metal ion [245]. A way to accomplish this is by binding of Ni^{2+} to His-tagged proteins. Due to high affinity of phosphopeptides towards Fe^{3+} ions, Fe^{3+} chelated columns are widely used for phopshopeptide enrichment [245, 246]. Unfortunately, acidic residues also bind to Fe^{3+} ions, leading to signaling interference by non-phosphopeptides. Converting the carboxylic acid groups to methyl esters reduces the interfering signal [243]. Other methods of enrichment also exist. Converting the phosphate group to stable thiol by β-elimination and capturing this thiol by column chromatography is one method [243]. There is another method where instead of directly capturing the thiol after β-elimination, converting the residue to an aminoethylcysteine renders it to be recognized by Lys targeting proteases like trypsin. This method shows higher quality of analysis [243]. Using matrix assisted laser desorption ionization time of flight (MALDI-TOF) gives better MS results as it improves the detection limit of phosphopeptides and helps to quantify the phosphorylation [247]. This technique is more sensitive and more reliable in identifying phosphor specific connexin residues. Both *in vitro* and *in vivo* samples have been used for MS analysis of specific phosphorylation residues [218, 248-250].

The second widely used modern method to identify phosphospecific residues is the use of highly specific antibodies that bind to phosphospecific amino acid residues [209, 236]. This method can be used along with MS as a way to validate the MS results. This method is the most used way of characterizing phosphorylation site specific physiological

functions for *in vitro, in vivo* and *ex vivo* studies [239, 251, 252]. As mentioned before, this method has been used to better identify the different electrophoretic bands of connexin proteins. For example, the slower migrating P1, P2 along with P0 have been linked to Tyr phosphorylation residues [252]. Since Cx43 is the most extensively studied connexin owing to its ubiquitous nature, many commercial phosphospecific antibodies exist for Cx43 residues. However, the same cannot be said for other connexins. Another drawback is that some commercial antibodies show some degree of non-specific binding, which can interfere with the specific bands while interpreting the data.

7.2. Ser/Thr phosphorylation

Phosphorylation of Cx31, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50 and Cx56 have been determined (as mentioned previously). But Cx43 is the easiest to study as it is endogenously expressed by almost all cell types. There are more commercially available reagents to study Cx43 and the reagents for Cx43 are of better quality. Thus, there have been more studies related to Cx43 phosphorylation, compared to others. The Cx43 has 39 residues capable of phosphorylation of which above 20 have been determined. Ser phosphorylation is the most well characterized, followed by Tyr and then Thr [157, 163, 209, 222, 253]. Very little is known about the functional relevance of Thr phosphorylation.

Many Ser kinases with Cx43 as substrate have been identified including MAPK, Akt, CaMKII, PKA, PKC, CDK1 [208, 250, 254-257]. The biological importance of Ser phosphorylation of Cx43 includes channel gating, channel trafficking, internalization. All these affect the GJIC. Kinases such as PKA, CK1 play roles in connexon trafficking towards the membrane, gap junction assembly and channel opening [165, 255, 258, 259].

On the other hand, MAPK, PKC, Akt, CDK1 and CAMKII phosphorylation of Cx43 induces channel closure and internalization, finally leading to degradation and turnover [208, 250, 254, 255, 257, 260]. Phosphorylation of newly formed Cx43 was detected when blocked in the ER-Golgi intermediate compartment (ERGIC) by brefeldin A, but the kinase was not identified. It was suggested that considering that many casein proteins are phosphorylated in the golgi, CK1 could have been the kinase involved [261]. CK1 phosphorylation of three Cx43 Ser residues (S325, S328, S330) have been shown to regulate gap junction assembly [165]. Phosphorylation of S373 and S364/S365 by Akt and PKA respectively promote trafficking towards the membrane [262-265]. Phosphorylation of S365 keeps the channel in the open state by preventing PKC phosphorylation of S368 [266]. Interaction of Ezrin (binds actin) with Cx43 in the perimembrane region promote further phosphorylation of Cx43 residues S364, S365 and S369 by PKA. This may cause Cx43 to interact with ZO-1 [267]. This ZO-1/Cx43 interaction said to keep the Cx43 near the gap junction plaque so that it can be incorporated into the plaque when required [268]. 14-3-3 protein has been shown to promote the engulfing of Cx43 at the membrane inside the vesicle [269]. Phosphorylation of S373 by Akt regulates the affinity of ZO-1 and 14-3-3 towards Cx43. It enhances association of 14-3-3 and dissociation of ZO-1 [270-272]. In the gap junction plaque, many Tyr kinases work to stabilize the Cx43 in the plaque.

Phosphorylation of Cx43 by the Tyr kinase Src initiates internalization [255]. Downstream of Src, MAPK phosphorylates S255, S279, S282 [220, 236, 239, 273-276]. Phosphorylation of the latter two residues causes partial channel closure and promotes binding of NEDD4 to Cx43. This leads to the formation of a clathrin coat around the connexin [106, 277, 278]. Concurrently, S365 is dephosphorylated providing access to

S368 which can now be phosphorylated, leading to channel closure [266, 279]. It is suggested that S365 phosphorylation causes a conformational change in the Cx43CT, that prevents PKC from accessing S368 [255]. The S262 phosphorylation by MAPK is required for total channel closure. This is suggested to happen simultaneously with PKC phosphorylation of S368 [150]. A study showed that phosphorylation of S255, S262, S279, S282 occurred simultaneously with S368 phosphorylation after treatment of cells with vascular endothelial growth factor [273]. Inhibiting PKC caused decreased phosphorylation of MAPK sites- S255, S262, S279, S282 and S368 (PKC site) suggesting MAPK phosphorylation is downstream of PKC [273]. CDK1 and CaMKII phosphorylation of Cx43 occurs in its end stage [280, 281]. CDK1 has been suggested to regulate gap junction internalization and reverse trafficking as a response to cellular stress or normal cell cycle needs [280, 282]. These phosphorylation events are connected to form a complex phosphorylation network. A phorbol ester phorbol myristate acetate called PMA, that activates PKC, was used to stimulate cells, it was seen that Akt phosphorylation of S373 (from 5-30 minutes) was highest at 5 min after PMA addition. This further decreased over time. Similarly, MAPK phosphorylation was seen to be induced at 10 min and was highest at 30 min. PKC phosphorylation increased at 10 min and was still high at 60 min. this study also showed that Src phosphorylation of Y247 followed a pattern similar to the PKC site [259]. This occurrence prior to gap junction turnover contradicts the previous finding that Akt phosphorylation increased gap junction plaque size. Two mechanisms were suggested to explain this phenomenon. One was that the increased membrane bound trafficking caused decrease in free Cx43 and another was that increased gap junction size decreased the energy requirements for internalization due to decreased dependency on membrane

invagination [259]. PMA treatment experiments have been used to study effect of Cx43 phosphorylation in wound healing [259, 283]. Similar phosphorylation order as described above with Akt, MAPK and PKC was observed [284-286].

7.3. Tyrosine phosphorylation

Although Ser phosphorylation is better characterized, more and more studies are slowly emerging that identify and explain Tyr phosphorylation of connexins. Compared to Ser kinases, very few Tyr kinases have been identified. Among these Tyr kinases, Src is the most common and the most well studied. Thus, I will discuss the phosphorylation of Cx43 by Src in details below, along with mentioning some other Tyr kinases. Tyr phosphorylation of Cx43CT is the focus of my work, which deals with role of two Tyr kinases (BTK and ITK) on Cx43CT phosphorylation.

7.3.1 Src phosphorylation of Cx43

pp60- Src (v-Src) was shown in earlier studies to cause a decrease in GJIC in the NIH3T3 cells [287]. Other Tyr kinases initially detected, such as v-Fps, are not well studied unlike Src. The structure of Src kinase consists of NT, SH3, SH2 and kinase domains. The NT is myristoylated and membrane bound and is highly conserved [288]. Activation of Src requires phosphorylation of Y416 in the kinase domain and autoinhibitory phosphorylation of Y527 in the CT domain [288]. The Y527 is missing in the v-Src, which is an oncogene, while the normal Src or c-Src has this residue. Cx43 residues Y247 and Y265 are known to be sequentially phosphorylated by v-Src. SH3 and SH2 domains are responsible for the interaction of Cx43 with v-Src [288]. The SH3 domain binds to regions of the substrate protein that are rich in Pro residues. Cx43 has a Pro rich sequence P274-P284 (with a PXXP

motif), that is necessary for interaction with SH3 domain of v-Src. This interaction causes the Cx43 to acquire a left-handed type II helical structure [289]. It also leads to the phosphorylation of Cx43 Y265, creating a docking site for the v-Src SH2 domain, which is followed by Y247 phosphorylation [290]. After co-transfecting HEK293 cells with v-Src and Cx43 Y265F, these two proteins could not be co-immunoprecipitated and the co-transfection also caused significant decrease in Cx43 phosphorylation. These studies show that Y265 is the main site of phosphorylation [291]. These observations may explain the higher presence of pY265 in gap junction plaques than pY247 [252].

In *Xenopus* oocytes, Cx43 phosphorylation by v-Src completely blocks Cx43 induced communication [291]. Src has also been shown to increase phosphorylation of Cx43 by MAPK (S279/282), PKC (S262/368) and decrease that by PKA (S364/ 365) [252]. This Src induced phosphorylation by other kinases is responsible for the rapid closure of gap junction channels [252]. Expressing Y324F, Y265F or Y247F/Y265F Cx43 mutants in Cx43 knockout mice showed resistance to GJIC disruption by v-Src [290]. In contrast to this, activation of v-Src led to disruption of the junctional conductance (3-6 hours post v-Src administration). The half-life of Cx43 in *Xenopus* being much higher than normal suggests that Tyr phosphorylation by v-Src is required for decreased GJIC in a chronic manner (within 24 hours) as opposed to an acute manner (within 4-5 hours) [290, 292]. It has been noted that Src induces MAPK and PKC to phosphorylate Cx43. This Src induced process is responsible for the rapid closure of gap junction channel.

Interaction of Cx43CT with other protein partners such as ZO-1, can be regulated by Src. It has been seen that Src disrupts the ZO-1/Cx43CT binding [268, 293]. NMR studies have shown that interaction of Src SH3 domain with Cx43 CT causes

conformational changes in the latter, leading to disruption of binding between PDZ-2 domain of the ZO-1 to the last residues of Cx43CT (S372-I382) [154, 289]. Recently, another Tyr residue Y313 has been shown to be phosphorylated by Src. Drebrin is a cytoskeletal protein that binds to Cx43CT. Phosphorylation at both Y265 and Y313 by Src causes disruption of interaction between Cx43CT and Drebrin [222]. Thus, to briefly summarize, binding of cytoskeletal elements such as Drebrin and ZO-1 to Cx43CT keeps the connexin in the gap junction plaque. Phosphorylation of Y247, Y265, Y313 by Src causes disruption of interaction between Cx43CT and cytoskeletal elements. This leads to internalization of connexins and as a result, decrease in GJIC [222].

7.4 Phosphorylation at motifs of other binding partners

Phosphorylation of Cx43 can change the secondary structure, alter channel pore size, selectivity, affinity of binding partners due to the introduction of the negative charge of phosphate group [255]. Certain phosphorylation sites have been shown to overlap with binding sites of other protein partners. This suggests that binding of the other proteins can be affected by phosphorylation [255].

In the forward trafficking process, Akt phosphorylation of Cx43CT causes ZO-1 to disassociate from Cx43 and 14-3-3 to bind to it [80, 270, 271, 294]. The Ezrin binding motif of Cx43 overlaps with PKA and PKC binding motifs. A different PKA binding residue just precedes this region, but studies showed phosphorylation of these residues did not affect Ezrin binding [263]. ZO-1 binding site overlaps with certain Ser residues, but this was also unaffected by phosphorylation [294].

In reverse trafficking (including disassembly and turnover), both Ser and Tyr phosphorylation play roles. Interaction of Cx43CT with ZO-1, Drebrin, β-tubulin stabilizes gap junction plaques [255]. These three cytoskeletal elements are all regulated by Src. Binding site of β-tubulin overlaps with Y247, a Src- phosphorylated residue. NMR studies using phosphorylated and non-phosphorylated Y247 Cx43, showed inhibition of β-tubulin binding due to Y247 phosphorylation [295]. Drebrin binding site overlaps with Y264 and Y313 [164, 222]. Phosphorylation of these tyrosines inhibits Drebrin binding. Reduction of Drebrin levels in the cell causes Cx43 internalization and reduced GJIC [222]. MAPK phosphorylation site is near the AP2 (clathrin complex protein) and NEDD4 ubiquitin binding sites (PPXY) [255]. Phosphorylation by MAPK doubled the binding affinity of NEDD4 for Cx43 [106, 107]. The PPXY site is also near to the AP2 and Tumor-susceptibility gene 101 (TSG 101) [296]. Binding of the latter two proteins regulates Cx43 internalization [296]. Phosphorylation by MAPK also influences phosphorylation by PKC. Phosphorylation of MAPK and Src sites causes phosphatase recruitment to dephosphorylate S365. This renders S368 to be phosphorylated by PKC [297]. Cx43 has three Tyr based sorting motifs (S1, S2, S3) that mediate its binding with AP2. S1 is near the plasma membrane. This causes steric hindrance preventing S1 from binding proteins. S2 overlaps with the PPXY site (for NEDD4). This means AP2 and NEDD4 both cannot bind to Cx43 simultaneously [298]. This also suggests that clathrin (AP2) mediated endocytosis and ubiquitin (NEDD4) mediated degradation do not occur simultaneously [298]. Using the yeast-two-hybrid system showed that the Y286A mutation in the S3 region caused the Cx43 mutant to no longer interact with AP2 [299]. Tyr phosphorylation of other proteins containing similar Tyr based sorting motifs inhibits AP2 binding [300]. This can

suggest that Y265 phosphorylation may inhibit AP2 binding. As the Y265 is a Src binding site, Src phosphorylation may affect AP2 binding [300].

8. Gap junction in the immune system

Over the last decade gap junctions have been shown to play major roles in the immune system. But, unlike other tissues, where gap junctions have been well studied, immune cells are usually avoided in context of studying gap junctions. This is because many immunological experiments are performed *ex vivo* with non-adherent cells. This makes the study of connexins difficult as they are mainly involved in cell-cell communication. However, this does not mean that connexins and gap junctions are not required for normal functioning. A set of connexins, such as Cx43, Cx30.3, Cx32, Cx37, Cx40 are expressed in immune and hematopoietic cells (expression of Cx43 is the most ubiquitous among these) [301]. Expression of the most important connexins in the immune cells and their corresponding roles are provided in Table 1. It is still unknown how their expression compares to those of other cells. In immune cells like human monocytes, expression of Cx43 increases after encountering signals for infections such as Lipopolysaccharide (LPS) and interferon (IFN-γ) [302]. This needs to be studied in further details to understand the downstream effects of the Cx43-mediated signaling. In the early 1990's it was shown that connexins can make functional gap junctions between immune cells [303-305]. Since immune cells are usually motile and can migrate to other locations such as lymph node after having visited other cells, they can transfer intracellular information obtained from gap junctional contact with residing tissue cells to other locations in an organism [305, 306].

Various cell types in the immune system have been shown to express connexins. These cells have varied functions by controlling different immune responses or controlling the same immune response in a different manner (for review, see [301]). For example, innate immune system consists of phagocytic cells such as monocytes/macrophages and dendritic cells (DCs). These cells express connexins and form functional gap junctions between identical as well as different cells [307, 308]. Most notably, connexins (especially Cx43) are expressed by almost all immune cells and can be upregulated when these cells get exposed to inflammatory factors. A few of the gap junction related communications in the immune cells are described below.

Table 1

Connexin expression in immune cells

Connexin isotypes	Cell types	Function	Coupled cell types
Cx43	Bone marrow (BM) derived DC	Cross-presentation DC activation	BMDC – tissue cells BMDC - bmDC
	Bone marrow stromal cells	Hematopoiesis	BM stromal cells - HSCs
	Follicular DC	Germinal center development	FDC – B cells FDC - FDC
	Monocytes	Cross - presentation	Monocytes – tissue cells
	Tonsil DC Appendix DC		DC – tissue cells
	Langerhans cells		Langerhans cells -tissue cells
	Macrophages	Inflammation	Macrophages – intestinal endothelium
	Thymus epithelial cells T cell progenitors	T cell development	TEC – T cell progenitor cells
	T cells B cells NK cells	Maturation Antibody secretion Activation	T cells- T cells T cells – B cells NK cells – NK cells
	Mast cells		Mast cells – mast cells Mast cells - fibroblasts
	Polymorphonuclear neutrophils (PMN)	Extravasation	PMN – PMN PMN - endothelium
Cx40	T cells B cells	Maturation Antibody secretion	T cells – T cells T cells – B cells / B cells – B cells
Cx37	Macrophages	Atherogenesis	Macrophages – smooth muscle cells
	Monocytes	Atherosclerosis prevention	Hemichannel
Cx32	Mast cells		Mast cells – mast cells Mast cells – surrounding cells
Cx30.3	Thymocytes		

Out of the 21 known human isoforms of connexin, Cx43 expression is the most ubiquitous in immune cells. In most cases, connexin expression can facilitate the formation of both homotypic and heterotypic gap junctions. GJIC plays important roles in the immune system (table is adapted and modified from [301]).

8.1 Connexins and gap junctions during hematopoiesis

Immune cells originate in the bone marrow. The bone marrow also has stromal cells. Both immune and stromal cells express Cx43. These two cell types interact with each other to facilitate Cx43 mediated intercellular communication in the bone marrow [309-313]. These immune stem cells can differentiate into all the different types of circulating blood cells, including all the members of cellular immune system. In the early hematopoieseis phase, Cx43 gap junctions seem to be critical to the terminal differentiation of primary B and T cells as shown in Cx43 deficient mice [310, 312]. In the hematopoietic system, stem cells can renew themselves. Connexin expression has been characterized in detail in the hematopoietic system. Earlier studies showed the expression of mRNAs of Cx30.3, 31, 31.1, 43 and 45 in bone marrow cells [309]. Analysis of freshly isolated bone marrow cells and studies conducted in derived cell lines proved that Cx43 was the major gap junction protein expressed by the specialized microenvironmental stromal cells and in stem cells [309, 314].

8.2 Connexins and gap junctions in lymphoid organs

T-cell lymphoid progenitors differentiate in the bone marrow. They then migrate into the thymus and form tight intercellular contact with stromal cells that aid in their maturation (for review, see [309]). The thymus consists of a complex network of cells. Thus, ensuring direct exchange of intercellular information is necessary. This provides the thymus a platform where T-cell clones can differentiate into matured circulating cells. Thymic epithelial cells and thymocytes form functional gap junctions of exclusively Cx43 [315, 316]. The function of these gap junctions is regulated by a variety of soluble factors such as growth hormones [316]. Clones of memory and effector cells migrate from primary

(thymic) to secondary (lymph node) lymphoid organs [309]. Here, they maturate further and differentiate upon antigen stimulation, a process which requires direct cell-cell contact. Other epithelial supporting cells and antigen presenting cells play crucial roles in this differentiation step [317, 318].

In the lymphoid tissue, Cx43 seems to be the only connexin. Studies have shown that Cx43 mRNA and protein expression are up regulated by antigenic stimuli [308]. Recently, studies have shown that Cx43 is important for antigen processing and presentation, which will be described below [308]. In the secondary lymphoid organs, follicular dendritic cells also play roles in antigen presentation. Dye transfer assays have indicated direct communication of these dendritic cells with each other and B lymphocytes [319]. It is proposed that gap junctions contribute to metabolism and development of germinal centers by controlling the growth of follicular dendritic cells [320].

8.3 Connexins as inflammatory response markers

A systemic or local response to infection is inflammation. The key components of inflammation include leucocyte migration, activation and maturation. Leucocytes interact with parenchymal cells and extracellular matrix. This helps the leucocytes to gain access to inflammatory sites. Another key process of inflammation is the transmigration of leucocytes across the endothelium [309].

Many studies have implicated connexins, especially Cx4,3 in the process of inflammation [309]. Smooth muscle and endothelial cells express Cx43 (the latter also expresses Cx37) [13]. Some *in vitro* studies have showed that co-culturing smooth muscle and endothelial cells caused increased production of pro-inflammatory cytokines such as

IL-1 and IL-6 and also higher expression of Cx43 [321]. Cx43 expression by leucocytes is also regulated by LPS stimulation and increases during ischemia reperfusion injury [303]. These studies suggest an effect of connexins on the overall inflammatory response in the arterial wall. Many studies have shown GJIC occurs between monocytes and endothelial cells by using dye transfer techniques [60, 322]. Lipid loading of monocytes and macrophages significantly reduced GJIC [323]. Cx43 expression is increased in regions of vessel intimal thickening where foam cells aggregate [60, 322]. By studying the expression patterns of Cx37, Cx40, Cx43, in low density lipoprotein (LDL) deficient mice as well as in human atherosclerotic plaques, redistribution of connexins in the arterial wall occurred during development of atheroma [324]. A study showed that using statins (drugs that decrease cholesterol biosynthesis by inhibiting HMG CoA reductase) reduced the Cx43 expression in cultured endothelial cells [325]. These studies suggest role of Cx43 in inflammation that leads to atheroma [309].

Connexin expression is also related to inflammation in other organs such as kidneys of patients with glomerulitis [326, 327]. Increase in connexin expression correlates with increase in Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Protein-1 (VCAM-1) following the invasion of inflammatory cells and damage of tubular and interstitial cells in the patients [326, 327]. Damage in the CNS and peripheral nervous system (PNS) also modified connexin expression [309]. In the CNS, roles of connexin hemichannels in astrocytes have been investigated in stroke models [328, 329]. In the PNS, Cx43 is majorly associated with perineural fibroblasts and Schwann cell cytoplasm [330-332]. In Schwann cells, there is higher expression of Cx32 at reflexive gap junctions (gap junctions between adjacent processes originating from the same cell) compared to

intercellular gap junctions [333]. In studies conducted using mouse and rat models of sciatic nerve damage, connexin expression is modified following the inflammatory response caused by peripheral nerve damage [334-336]. Injury to peripheral nerve causes changes in Cx32 and Cx46 expression [335, 336]. There has been found to be a link between downregulation of Cx32 and upregulation of Cx43 in endoneural fibroblasts and infiltrating macrophages [321, 337]. It is proposed that these may be induced by release of pro-inflammatory cytokines by endothelial cells and transmigrating leucocytes [321, 337]. Change in Cx32 and Cx43 expression varies among different tissue types. LPS induced inflammation in liver causes rapid degradation of Cx32 [102, 338, 339]. This correlates with a decrease in GJIC as noticed by lower degree of dye transfer [340].

8.4 Connexins and gap junctions in the innate immune system

When we encounter pathogenic infection, the innate immune system provides the first line of defense. This system consists mainly of phagocytic cells such as macrophages and neutrophils as well as dendritic cells and natural killer (NK) cells. These cells act in an integrated manner to remove infectious agents [341, 342]. Many studies have characterized the role of gap junctions in innate immune cells. GJIC has been shown between two macrophages, between macrophages and intestinal epithelial cells [343-346]. Electron microscope studies have shown bone marrows have gap junction like structures in contacting macrophages, between macrophages and reticular cells, neutrophils, eosinophils, monocytes and erythroblasts [347]. These studies also indicate role of connexin in hematopoiesis. It has been confirmed that Cx43 is the constituent protein of macrophage gap junction [302]. Many studies have focused on the regulation of these gap junctions by different pro-inflammatory factors such as cytokines and growth factors [302,

348, 349]. Connexins play key roles in polymorphonuclear neutrophil functions after these cells are activated by lipopolysaccharide *in vivo* and *in vitro* [303]. Other studies have also corroborated these findings, where they have identified gap junctions at leucocyte-leucocyte and leukocyte-endothelial cell contacting regions. gap junctions have also been implicated in the trans-endothelial migration of these cells and their modification under inflammatory conditions [60, 302, 303, 308]. Both Cx40 and Cx43 have been found to be expressed in these cells [350]. Coupling of endothelial cells to neutrophil via gap junctions occurs in a time dependent manner, which is regulated by the cytokine tumor necrosis factor (TNF) and is facilitated by heterotypic cell adhesion, which also involves many adhesion molecules [350].

Mast cells, which are located in the mucosa and connective tissue, account for less than 0.2% of total leucocytes. Their proliferation requires direct interactions with T-lymphocytes [351]. These cells play major roles in allergic interactions and reactions and provide immunity against parasitic infections [309]. Electron microscopy studies show that gap junctions connect mast cells and fibroblasts in a chick embryo model [352]. These gap junctions appear to be consisted of Cx32 and Cx43 [352]. These studies strongly indicate roles of gap junctions in immune tissue microenvironments.

The DCs, which are also termed as the ‘master regulators’ of the immune system, also express connexins that gap junctions to interact with their environment [301, 302, 307]. After detecting inflammation, DCs as well as human monocytes show upregulation of Cx43 and form gap junction channels [302, 307]. This suggest that the DCs contact the environment to sample electronic or metabolic information from neighboring cells in response to infection [307]. Many different tissues express different members of the DC

family, such as the liver DCs, skin DCs, intestinal DCs, which interact with their environment. It has been shown that communication between DCs is required for their activation, but the signaling process remains to be elucidated [308, 353].

8.5 Connexins and communication in lymphocytes

In the early 1970's, with the help of electrophysiological studies, low intercellular resistance properties were demonstrated to indicate the presence of gap junctions between contacting lymphocytes [354, 355]. After this discovery, there was a lack of interest in this study for over two decades because it was thought that gap junctions will not be important in lymphocytes as these cells spend majority of their time migrating. However, later several studies have indicated the high significance of gap junctions in lymphocyte contact. Purified peripheral B cells, T cells and NK cells from healthy humans have shown to express Cx43 [305, 356]. Lymphocytes derived from secondary lymphoid organs also express Cx40 [305]. Flow cytometry studies using anti-peptide connexin antibodies binding to the extracellular loops showed connexins to be located on the cell surface [305]. These studies also highly suggest the presence of unopposed hemichannels on lymphocytes with independent communication functions such as ATP-mediated propagation of calcium waves [357-359].

Calcein, which is a gap junction permeable fluorescent dye, is used to study gap junctions in non-adherent cells. Calcein was observed to be transferred between contacting lymphocytes. This direct transfer was also seen to be inhibited by two gap junction inhibitors, thus, implicating direct communication across gap junctions in these cells [305]. An inhibitor of gap junctional communication called 18-α-Glycyrrhetinic acid, was shown to cause modification of protein kinases that participate in the regulation of Cx43 [360,

361]. These Cx43 are highly phosphorylated at multiple Ser residues and a few Thr residues on the CT domain [220]. In contrast to this, the connexin mimetic peptides which bind to specific amino acid sites on the EL of Cx43, do not penetrate into cells. This accounts for their high specificity and indicates cellular metabolism is absent [362]. Thus, connexin mimetic peptides are widely being used to study gap junctions in the immune system. Not just gap junctions, but hemichannels too are being studied using connexin mimetic peptide tools [358, 359, 363]. Exposing lymphocytes to GAP26 and GAP27 (which are short peptides that correspond to specific amino acid sequences in the extracellular loops of Cx43) for 10-50 hours, significantly reduced the production of immunoglobulins (Ig) A, G, M in mixed cultures of human B and T lymphocytes [356]. Rectification in homotypic and heterotypic channel function to a small degree in B and T cell cultures suggest an importance of lymphocytic gap junctions in the polarization of immune response [356]. Introduction of connexin mimetics to cell media also showed complicated temporal inhibitory effects on the production of cytokines (but lesser effects on cytokines IL-2 and IL-10) [305, 356]. Fluorescence activated cell sorting (FACS) analysis using antibodies against EL of Cx43 in different lymphocyte types (CD4+, CD8+, CD19+ cells) showed differences indicating that these cell types have their characteristic surface patterns under mitogenic stimuli [305].

Interaction between lymphocytes and endothelial cells is necessary for the normal functioning in context of vascular physiology [309]. Lymphocytes migrating in the blood leave the circulation via specialized post capillary vessels called the high endothelial venules [364]. This active method of transmigration across the endothelial cell layer is complicated and requires a chain of time-dependent molecular mechanisms that guarantee

not just adhesion but also direct intercellular communication [364]. Presence of gap junctions occurs at varying degree in the vascular system. Cx37, Cx40, Cx43 have been consistently found to be present in the vascular walls [306, 365, 366] as shown by mRNA and protein studies. Lymphocytes form functional gap junctions *in vitro* with endothelial cells during the active process of transendothelial migration in a time sensitive manner. Connexin mimetic peptides also caused inhibition of this process [367].

B and T cells develop and mature in the thymus and lymph nodes. In the thymus, progenitor T cells contact with the surrounding epithelial cells and DCs. These cell types express Cx43. Cx43 is required for proper maturation of T cells as shown in Cx43 deficient mice [311]. However, the molecular mechanism involved in this process is unknown. In the secondary lymphoid organs such as lymph nodes, T cells encounter antigens, processed and presented by DCs. T cells that survive the process of negative selection in the thymus then migrate into the peripheral tissue spaces where they may encounter antigenic peptides. These T cells express Cx43, which may allow communication with surrounding tissues which is required for proper activation and differentiation.

Expression of Cx43 is also found in B cells. B cells interact through gap junctions with follicular dendritic cells in the secondary lymphoid organs. This cell coupling may help synchronize germinal center processes and facilitate the transfer of anti-apoptotic molecules. This helps to rescue the B lymphocytes from apoptosis [308]. The B cells, T cells and NK cells isolated from tonsils have low expression of Cx40, and the function of Cx40 gap junctions in these cells requires further studies. Cx40 has been suggested to form hemichannels in these cells, which facilitate ATP-mediated propagation of calcium waves [305, 368] as mentioned earlier. B cells need to produce antibodies and T helper cells help

in this process. The role of gap junctions in mixed lymphocyte culture has been studied and shown that GJIC is required for proper and efficient antibody secretion by the B cells. This study indicates that GJIC in B cells is needed for optimal antibody secretion [367]. Recent studies have also shown B cell-B cell interaction via gap junctions for their proper maturation [301].

8.6 Connexins in antigen presentation

As mentioned earlier, gap junctions allow particles of approximately 1 kDa to pass through them. Interestingly, many pathogen particles that need to be presented antigenically to get neutralized, also fulfill this criterion [301, 369]. Fragments are presented by MHC class I molecules in the form of peptides of only about 9amino acids (around 1 kDa) to cytotoxic T cells (CTLs) [369]. These CTLs then work by killing infected cells in response. The majority of the peptides to be presented, are derived from proteins that are degraded in the cytosol. The processes required before antigenic presentation is extremely complicated. After successful processing, the fragment of intracellular protein is then presented as a peptide by MHC class I molecules [369, 370]. Peptides upto 1850 Da (about 16 amino acids) can be transferred through gap junctions, which is larger than most of the peptides presented by class I MHC molecules [371]. These peptides appear much stretched compared to other shorter peptides. This exception in the gap junction cut-off limit can be attributed to the lack of secondary structures in the peptide fragments. They are usually flexible linear strands, which can diffuse freely through gap junctions, as opposed to a small molecule with a more complicated 3D structure [307, 371]. This has been demonstrated by comparison of Cx43 mediated intercellular diffusion in a linear vs a circular 8-mer peptide of same sequence. Only the linear peptide showed

diffusion through gap junction, indicating the importance of 3D structure in determining gap junction passage of molecules [307, 372]. Thus, gap junctions connect the antigen processing machinery of two connecting cells allowing immunological coupling. This process can also cause the killing of non-infected cells by cytotoxic T lymphocytes when these cells end up acquiring infection peptide fragments from neighboring infected cells [372]. The elimination of non-infected cells can be helpful in case of viral infections to prevent further infections [372, 373]. Gap junction mediated peptide transfer causing immunological coupling also allows antigen presentation by cells other than the infected ones [374-376]. This suggests a novel function of gap junctions in immunological coupling.

8.7 Connexins in cross presentation

For an effective immune response to occur as a result of infection, a complex network of processes is required. Proper activation and expansion of specific cytotoxic T cells are parts of these processes. Antigen presenting cells (APCs) include DCs and macrophages and are responsible for the activation of T cells [377]. APCs acquire information from the infected cells and transfer this information to lymph nodes to specifically stimulate CTLs for activation and expansion by antigen presentation, aided by expression of co-stimulatory molecules [377, 378]. Transfer of antigenic information includes a process called cross-presentation [378-381]. DCs and activated monocytes express Cx43 and can form gap junctions with other cells [302, 353]. It has been seen that activated monocytes can acquire antigenic information from influenza- infected cells in the form of peptides through gap junctions [307]. These GJs allow immunological coupling through cytosolic transfer of antigenic peptides for cross-presentation by DCs and activated

monocytes [307]. Post this gap junction mediated coupling, tissue DCs can sense immunological, metabolic and electrical alterations in their surroundings, and can respond by activation and by migrating to lymph nodes and also by cross-presentation of antigenic information acquired through gap junction mediated communication. The MHC class I molecules, which present peptides on the cell surface, provide the immune system with a sample of the protein content of a cell. Proteins that inhibit MHC class I presentation, are expressed by viruses. This also prevents cross-presentation [382]. Human Papilloma virus 16 and Herpes Simplex virus 2 express proteins that have been shown to block gap junctional contact. This possibly prevents cross-presentation, which ultimately reduces their chances of discovery by the immune system [383, 384].

9. Importance of BTK in the immune system

Bruton's Tyrosine Kinase is a non-receptor Tyr kinase that belongs to a family of Tec kinases. Its initial discovery was in 1952 when Ogden Bruton first identified X-linked agammaglobulinemia (XLA) [385]. About four decades later, two separate studies identified a mutation in BTK to be linked to XLA [386, 387]. In XLA patients, B cell development in the bone marrow is hampered and in severe patients, it leads to almost no functional Igs in the serum. This increases their susceptibility to diseases as they have very little antibody to neutralize antigens [388, 389]. BTK is now known to play key roles in many biological processes such as B cell differentiation. BTK is phosphorylated upon B cell antigen receptor stimulation. This causes activation of BTK, and thus increases its kinase activity [390]. Similar studies have implicated BTK in B cell receptor (BCR) signaling. By blocking the BTK expression in mice, there is decrease in the number of matured B cells along with Ig deficiency in the serum [391]. XLA is a more severe form

of BTK deficiency. In X-linked Immunodeficiency (XID), which is a milder form, BTK has a point mutation. Even though the symptoms are milder than in XLA, BCR mediated signaling is still disrupted [392].

9.1 BTK in signaling pathways

One of the first studies to establish a link between BTK and BCR signaling showed BTK to be downstream of the antigen receptor [390, 393]. Activation of BCR is followed by a chain of signaling events that result in activation, differentiation and proliferation of the B cell. The BCR includes IgM heterodimers, which bind to antigens on the B cell surface and initiate B cell activation [389, 394]. The tail region of the IgA and IgB has a tyrosine based immunoreceptor activation motif. Following BCR stimulation, the Plekstrin Homology (PH) domain of BTK associates with the plasma membrane and Y551 in the BTK kinase domain is transphosphorylated by Syk, Lyn or other kinases of Src family. Phosphorylation of Y551 promotes the autophosphorylation of Y223, located in the SH3 domain [389, 395]. Inhibiting this Y223 phosphorylation can block full activation of BTK [395, 396]. BTK activation is followed by downstream signaling pathways that include other Src family kinases besides many other non-Src family kinases such as MAPK and PKC [397, 398]. Activated BTK phosphorylates phospholipase Cγ (PLCγ2). This is followed by cleavage of two second messengers, Diacylglycerol (DAG) and Inositol triphosphate (IP3) by PLCγ. This regulates downstream proteins such as MAPK and nuclear factor of activated T cells [398]. Many recent studies about the role of BTK in BCR signaling have been done in lymphoma cells. Autoantigens cause B cell receptor mediated activation of NF-κB through processes that include BTK, Syk, PKCB and promotes recruitment and assembly of an adaptor complex required for B cell signaling [399-401].

APCs express Toll-like receptors (TLRs) that can induce adaptive immune response [402]. TLRs and BCRs together control immune response against infection by creating a link between the innate and adaptive immune response. Recruitment of Myeloid Differentiation Primary Response 88 (MYD88) is required for TLR signaling in B cells [403]. BTK has been shown to interact with MYD88 and other proteins downstream in the TLR signaling pathway [404]. Combinatorial targeting of TLR and BTK is being predicted as a therapeutic target in chronic lymphocytic leukemia [405]. This TLR/BTK crosstalk has also been demonstrated in autoimmune diseases [406]. Besides TLR signaling, chemokine receptor signaling is another pathway where the role of BTK has been demonstrated. Stromal cell derived factor-1 (SDF-1), which is an activator of BTK, is required for CXCR4 (a chemokine receptor)-mediated B cell processes such as B cell trafficking from peripheral blood to lymphoid tissues [407]. Another chemokine receptor (CX3CR1) is linked to immunosuppression. Defects in actin remodeling, a process that is usually controlled by CX3CR1, caused impaired BTK expression and BCR signaling [406].

9.2 Role of BTK in other cells

BTK plays key roles in the functioning of cells other than B lymphocytes. Besides B cells, BTK expression is high in macrophages, mast cells, DCs, whose main functions are to eliminate pathogens [408-411]. Thus, BTK is expressed in both innate and adaptive immune cells.

The *in vivo* inhibition of BTK has been shown to increase the persistence of activated T cells along with decreasing Treg/CD4+ T cell ratio. This reduces the immunosuppression caused by CLLs via both BTK dependent and independent methods

[412]. Ibrutinib (BTK inhibitor) therapy increases CD4+ and CD8+ T cell numbers. Ibrutinib also increases the anti-tumor properties of T cells [412, 413]. In another study, ibrutinib showed improvement in CD8+ T cell function in CLL [414].

BTK is also linked to macrophage function. A study showed that BTK inhibition suppressed FcγR-mediated cytokine production. This effect was rescued by interferon (IFN) priming after co-culturing monocytes with NK cells *in vitro* [415]. Ibrutinib inhibits BTK in Nurse-like Cells (NLC). This reduces the phagocytic ability and enhances immunosuppression related to NLC's expression of M2 markers [416].

BTK also affects neutrophil function. A neutrophil/BTK signalosome is shown to activate macrophage-1 (Mac-1), and thus increases neutrophil recruitment during inflammation [417]. In mast cells, BTK plays a role in FcεRI-mediated signal transduction [418]. An inflammasome complex called NOD-like receptor protein 3 (NLRP3) is essential for IL-1 related diseases [419]. BTK directly enhances the NLRP3 activity, indicating another importance of BTK in innate immunity [420].

9.3 BTK and diseases

Recent studies have also demonstrated the role of BTK in fungal, bacterial, and viral infections such as SARS-CoV-2 virus [408, 421, 422]. Defective BTK signaling in macrophages increases susceptibility to pulmonary aspergillosis, a fungal infection affecting the respiratory system [423]. BTK inhibition by ibrutinib severely disrupts macrophage response to *Mycobacterium tuberculosis* [424]. Role of BTK was studied in BTK-deficient pneumosepsis model, and the results showed that BTK helps in maintaining vascular integrity in the lung [406]. Recently COVID-19 patients who showed

hyperinflammation and increasing dependence on oxygen, were treated with the second generation BTK inhibitor acalabrutinib, and showed rapid improvement in oxygen concentration and inflammation [425]. This may be caused by reducing the B cell mediated immune response in COVID-19 as hyper immune response is a prognosis in the severity of this disease. However, it should be considered that inhibiting BTK has effects on innate and adaptive immune responses. Besides pathogenic infections, BTK also has important roles in rheumatoid arthritis, multiple sclerosis, lymphomas among others [426-429].

10. Importance of ITK in the immune system

The first experimental studies on ITK (Interleukin-2-Inducible T-cell Kinase) were conducted in the late 1980s and early 1990s [430-433]. ITK expression has been found in T lymphocytes. Similar to BTK, ITK is also a non-receptor tyrosine kinase belonging to the Tec family of kinases. Its structure and cellular mechanisms are also similar to those of BTK. ITK activation begins with recruitment of its PH domain to the cell membrane [434, 435]. This is followed by a series of events that leads to phosphorylation of Y511. This in turn leads to autophosphorylation of Y180 in the SH3 domain [435]. The downstream substrate of ITK is PLCγ1 (for BTK it was PLCγ2) [435, 436]. There is a Tec homology (TH) domain in ITK just after the PH domain. This TH domain has a proline rich motif, whose binding to the SH3 domain of ITK causes inhibition of the kinase activity [436, 437]. ITK mediated T cell signaling has downstream effectors in the form of many proteins that include MAPK and PKC, like BTK [438, 439].

ITK plays a major role in T cell signaling. It has been seen that naïve T cell activity is impaired due to absence of ITK [436]. ITK plays the central role in a complex called the LAT-SLP-76 complex which itself is important in T cell signaling [440]. Studies using

ITK knockout mice showed impaired T cell activation in response to TCR mediated signaling [441]. ITK regulates T cell differentiation and its effector functions. ITK deficient mice have been shown incapable of generating TH2 response to *Leishmania major* infection [442]. Significant increase in TH2 response to this disease is noticed in WT mice [442]. Instead of TH2, lack of ITK was associated with a protective TH1 response that mitigated the infection. This study and similar ones done *in vivo* and *in vitro*, showed the role of ITK in producing a TH2-mediated signaling response [442, 443].

In the T cells activated specifically in response to parasites, analyzing the cytokine responses showed that T cells of ITK knockout mice produced less IL-4, IL-5, and IL-10 [444]. Similar *in vitro* studies also showed CD4+ T cells to be stimulated under TH2 differentiating conditions compared to WT mice [445]. Global gene expression analysis has shown that in ITK knockout mice, CD4+ T cells are incomparable to that of WT before activation [438]. Thus, it may explain why they respond differently than naïve T cells to parasite infections. Infecting ITK deficient mice directly with pathogens have indicated that ITK is not required for initiating TH2 response, but the effector T cell response was impaired [446].

10.1 ITK and diseases

Studies have indicated the involvement of ITK in inflammatory diseases such as allergic asthma, atopic dermatitis, aplastic anemia, peripheral T cell lymphomas. Itk involvement also occurs in promoting neuroinflammation.

In allergic asthma, there is contrasting results on role of ITK. Few studies have shown that loss of ITK results in reduced airway inflammation due to less infiltration of

TH2 cells after allergen treatment in pulmonary lung tissues [446]. Other studies showed that using ITK inhibitors did not reduce inflammation after rechallenging the mice with allergens [435]. Since the number of T cells and TH2 cytokines increased in the airways, the authors suggested that inhibiting ITK in asthma patients would not be therapeutic [447].

In atopic dermatitis patients, high levels of ITK were found in peripheral blood T cells [448]. In models of acute contact hypersensitivity reactions, ITK knockout mice have beneficial effects showing reduced inflammation [435, 449]. Studies in human cells have shown that inhibiting ITK leads to a reduced IFN-γ and IL-2 production combined with reduced cell proliferation [449]. However, the molecular mechanisms by which ITK induces inflammation is unknown.

In aplastic anemia, hematopoietic cells are destroyed, and thus makes the bone marrow empty. T cells have been implicated in this hematopoietic destruction [450]. ITK is involved in the activation of the transcription factor T-bet [451]. Aplastic anemic patients showed high level of T-bet, thus suggesting involvement of ITK in the inflammatory response [451]. This study also showed correlation of T-bet levels with those of ITK.

ITK has been implicated in inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Inhibiting ITK along with Plant receptor like kinases (RLK) prevents TH1 differentiation, and thus in turn prevent colitis development [452]. This suggests ITK's role in promoting inflammatory bowel disease. However, data regarding involvement of ITK in IBD have yielded contradictory results. ITK also plays a key role in neuroinflammatory diseases such as autoimmune encephalomyelitis [453]. By decreasing the transmigration of CD4+ cells into the CNS and across the blood endothelial

barrier, ITK depletion decreases the severity of the disease. This was also correlated with reduced secretion of TH1 and TH17 effector cytokines [453].

Loss of ITK function due to mutation can lead to Epstein Barr Virus associated lymphoproliferation [454-457]. ITK mediated severe immune dysregulations may lead to non-Hodgkin lymphoma, lymphoproliferative disease, mononucleosis, dysgammaglobulinemia, and hemophagocytic lymphohistiocytosis [458-462]. R335W mutation in the SH2 domain of ITK destabilizes the SH2 domain and has been found in EBV as well as Hodgkin lymphoma patients [454, 463].

11. Objective

Src kinase has been shown to phosphorylate Y247, Y265, Y313 in the Cx43CT. These phosphorylation events lead to disassociation of cytoskeletal elements such as Drebrin, ZO-1, β-tubulin from Cx43CT. The disassociation destabilizes Cx43 at the plasma membrane and promotes internalization of connexin. This decreases GJIC.

An *in vitro* kinase screening assay was done by Dr. Li, a previous member in Dr. Sorgen's lab. BTK and ITK were two novel kinases that showed to phosphorylate Cx43CT domain. Both these kinases share structural and functional similarities with Src kinase. They are all non-receptor Tyr kinases. They have similar structural domains – PH, SH3, SH2, kinase domains. Their downstream signaling mechanism also involves common proteins including MAPK, Akt, PKC.

Due to these similarities, BTK and ITK may also phosphorylate Y247, Y265, Y313 residues in Cx43CT. This phosphorylation will lead to decreased GJIC. Since, B cells (expressing BTK) and T cells (ITK) require to be migrated to target tissues to elucidate immune response, decreased GJIC in these cells may cause them to separate from other contacting cells, and render them more motile, so that they can migrate in response to immunological stimuli. Since B and T cells have little-to-no expression of Src, BTK and ITK may be compensating for Src in B and T cells respectively.

The work in this dissertation shows the effect of BTK and ITK on Cx43CT phosphorylation, their localization in cells after their activation along with localization changes in Cx43, effect on Cx43 mediated GJIC in cells overexpressing BTK or ITK and cells with endogenous expression of BTK or ITK.

CHAPTER TWO

Role of Bruton's tyrosine kinase (BTK) in regulating Cx43 gap junctional intercellular communication

The bulk of data in this chapter has been published in Basu et al.

Biomolecules, 2023 [464]

12.1 Introduction

Connexins are four-pass transmembrane proteins with cytoplasmic domains (N-terminal, cytoplasmic loop, C-terminal) and extracellular loops [21-26]. Six such connexins oligomerize to form a connexon. Connexons are trafficked towards the plasma membrane and get integrated into the membrane [78]. One connexon from one cell can come in contact with a connexon from another cell and form a gap junction channel that allows ions and small molecules to pass through [1, 2]. Thus, a gap junctional intercellular communication (GJIC) is established. There are various factors that regulate the GJIC. Of these, one major factor is the phosphorylation of Tyr residues in the C-terminal of Cx43 by protein kinases. Phosphorylation of Cx43 residues Tyr 247 and Tyr 265 by Tyrosine Kinase 2 causes decrease in GJIC by increasing the turnover rate of the connexin [250]. Phosphorylation of Tyr 247, Tyr 265, Tyr 313 of Cx43 by Src kinase disrupts the interaction between C-terminal of Cx43 and cytoskeletal elements such as Drebrin, ZO-1. This also leads to a decrease in GJIC [222].

By far, Cx43 is the most ubiquitous connexin expressed in human tissues [465]. The role of Cx43 has been implicated in various processes involving B lymphocytes. The CT domain of Cx43 is important for B cell spreading [466]. It also provides a scaffold for various protein interactions, which are involved in B cell signaling processes [467]. Cx43 is involved in hematopoiesis and is thus important for B cell formation. Heterozygous Cx43 mice exhibit defect in B cell development [312]. In splenic B cells, Cx43 helps in their adhesion to the surrounding cells [468].

Recently, a kinase screening was done where purified C-terminal of Cx43 was screened for phosphorylation by different kinases. BTK (involved in B cell activation) was

one of the kinases to give a positive hit, indicating that this kinase phosphorylated the C-terminal of Cx43. Following this kinase screening data, our lab had a mass spectrometry analysis done, which revealed four Tyr residues to be phosphorylated by BTK. Based on these observations, we studied the effect of BTK on GJIC through phosphorylation of the C-terminal of Cx43.

12.2 Materials and Methods

Antibodies and Detection Reagents

α-Vinculin (#4650S, Western blot dilution [WB] - 1:1000), α-BTK (#8547S, WB - 1:1000, Immunofluorescence [IMF] - 1:200), α-phospho-BTK (#5082S, WB - 1:1000, IMF - 1:100), α-rabbit-Alexa 488 (#4413, IMF - 1:500), α-mouse-Alexa 647 (#4410, IMF - 1:500), anti-rabbit IgG (#7074S, WB - 1:5000), anti-mouse IgG (#7076S, WB - 1:5000) were purchased from Cell Signaling; α-Cx43 (#C6219, WB - 1:2000, IMF - 1:500); Cx43 pY247 antibody was custom made from LifeTein and α-Actin (#A5441, WB - 1:5000) were purchased from sigma; Lucifer Yellow (#L453, 2.5 mg/ml, was purchased from Life Technologies); and DAPI (#5748) was purchased from Tocris. F(ab')2-Goat anti-human IgM (referred hereafter as α-IgM) was purchased from Invitrogen (#A24484).

Cell Culture

HeLa cells (gift from Dr. Steve Caplan, University of Nebraska Medical Center) and HEK293T cells (gift from Dr. Myron Toews, University of Nebraska Medical Center) were cultured in Dulbecco's modified Eagle medium or DMEM (from Corning) in a 37˚C incubator with a humidified 5% CO_2 atmosphere. The DMEM was supplemented with 2mM L-glutamine (HyClone), 0.2% Normocin (Invitrogen), 1% Penicillin-Streptomycin (from Corning) and 10% fetal bovine serum. Daudi cells (#CCL-213 from ATCC) were

cultured in Roswell Park Memorial Institute Medium (RPMI) 1640. RPMI 1640 powder (ThermoFisher) at 0.84% was added to water and this solution was further prepared to contain 25 mM D-Glucose, 17.85 mM Sodium Bicarbonate, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM Sodium Pyruvate, 10% fetal bovine serum and 1% Penicillin-Streptomycin. The pH of this medium was 7.4.

Transient Transfection

HEK293T cells were grown in DMEM up to 75% confluency. Transfection of these cells with the plasmids of interest (at 2.5µg/ml) was done using X-tremeGENE and OptiMEM. They were maintained for approximately 16 hours prior to lysis for Western blot or fixing for immunofluorescence.

Stable Cell Line Generation

HEK293T cells (HEK^{Par}) were grown in DMEM up to 60% confluency. These cells were transfected with BTK plasmid (at 2.5 µg/ml) under antibiotic free condition and cultured for another 72 hours. The media was then changed to selection media which contained 2 µg/ml puromycin. Cells were further maintained to obtain monoclonal cell lines, which was achieved by preparing cloning disks made from Whatman paper. Clones were finally screened and selected by comparing Western blot and immunofluorescence data (referred to here as HEK^{BTK}).

Cell Lysis for Western Blot

Cultured cells were rinsed with 1x Tris-Buffered Saline (TBS) prior to lysis. Lysis buffer was added to HeLa and HEK293T cells, followed by scraping these cells from the plate with a cell scraper. The scraped cells were transferred to a centrifuge tube and kept

on ice for 30 minutes after subjecting them to sonication for 10 seconds.Daudi cells were pelleted down (at 125 *x g*, 8 minutes) after rinsing with 1x TBS. The pellet was resuspended in lysis buffer and kept on ice for 30 minutes. The lysis buffer contained protease/phosphatase inhibitor buffer, 2 mM Phenylmethylsulfonyl fluoride (PMSF), 2 µM pepstatin A. The cell suspensions kept on ice were centrifuged at 1500 RPM for 10 minutes at 4˚C and the lysates (the supernatant) were removed from the pellet and kept on ice. These lysates were quantified by bicinchoninic acid assay (BCA) and normalized to a final concentration of 2 mg/ml protein.

Western Blot

Confluent cells were washed in 1x TBS and lysed in a buffer containing protease and phosphatase inhibitors, 2 mM PMSF, 2 mM Pepstatin A, and 1% Triton X-100. Bicinchoninic acid assay (BCA) was used to measure the concentration of lysates. 6x loading dye (69% 4x Tris-Cl/SDS, 29% glycerol, 0.3 M SDS, 0.6 M DTT, 0.19 mM bromophenol blue, dissolved in water, pH 6.8) was added to the lysate and 30 µg of protein was loaded on a 10% SDS-PAGE gel, followed by transfer to PVDF (Millipore) membrane. Blocking of membrane was done in 5% bovine serum albumin (BSA)/TBS with tween-20 for 1 hour before primary antibody incubation overnight. The membrane was washed in TBS with tween before secondary antibody incubation for 1 hour. Signal detection was done with SuperSignal West Femto (Thermo Scientific) substrate using digital imager (iBright, Thermo Fisher Scientific).

Immunofluorescence

Cover slips (Neuvitro) were coated with fibronectin (solution made in 1x PBS at 1:50 ratio) and HEK cells were seeded on them overnight to reach 98% confluency. For the Daudi cells, PLL coated cover slips were used. The cover slips were transferred to 24 well plates. (1/well) and rinsed with 1x PBS. 300 µl of fixing solution (3.7% formaldehyde, 0.3% TX-100, 1x PBS) was added to each cover slip and incubated at room temperature for 30 minutes. They were rinsed with 1x PBS twice for 10 minutes each time and incubated with immunofluorescence blocking buffer (1% bovine serum albumin (BSA), 0.3% TX-100, 1x PBS) at room temperature for 1 hour. Primary antibodies were prepared in immunofluorescence blocking buffer and added to the blocked cover slips overnight at 4˚C. Next day, the excess primary antibody was removed, and cover slips washed with 1x PBS twice for 10 minutes each time. This was followed by incubation with secondary antibodies (dissolved in immunofluorescence blocking buffer) at room temperature for 1 hour and washing with 1x PBS for 10 minutes. DAPI (4',6-diamidino-2-phenylindole) in 1x PBS was added and incubated for 10 minutes at room temperature, followed by rinsing with 1x PBS for 10 minutes. Cover slips were mounted on glass slides with SlowFade antifade. Excess antifade was blotted off and cover slips were sealed and dried before being imaged with confocal microscopy.

Triton X-100 Solubility Assay for Western Blot

Cultured cells (in 10 cm plates) were rinsed with 1x TBS. 1 ml lysis buffer (protease inhibitor cocktail, 2 mM PMSF, 2 µM pepstatin A) was added to the cells. The cells were scraped off with a cell scraper and transferred to a 2 ml centrifuge tube. Lysis was done by sonication for 10 seconds and lysates were quantified by BCA method. Following

quantification, samples were normalized to 1.5 mg protein in 1ml solution. 10% TX-100 was added (final concentration-1%) and sample was incubated at 4˚C for 1 hour on rotator. Samples were then vortexed prior to separating them for fractionation. From the 1ml sample, 450 µl was separated and mixed with loading dye and reserved as total protein. Another 450 µl was transferred to ultracentrifuge tubes and fractionated by ultracentrifugation at 100,000 *x g* for 1 hour at 4˚C. Supernatant was carefully removed, mixed with loading dye and reserved as soluble fraction. 450µl of solubilization buffer (8 M urea, 2.5% SDS, 0.1 M dithriothreitol (DTT), protease inhibitor cocktail, 2 mM PMSF, 2 µM pepstatin A) was added to resuspend the pellet, then mixed with loading dye and reserved as insoluble fraction. The fractions were kept at 4˚C overnight prior to Western blot analysis.

***in situ* TX-100 Assay**

Cultured cells were seeded on cover slips up to 98% confluency and washed with 1xPBS. Cells were incubated with cold TX-100 buffer (1x PBS, 1% TX-100, protease/phosphatase inhibitor, 1 mM $CaCl_2$, 1 mM $MgCl_2$) at 4˚C for 30 minutes. Following incubation, cells were washed with 1xPBS twice, then fixed and immunostained.

Scrape Loading Dye Transfer Assay

Scrape loading of HEK-293T cells was done as described previously [469]. All reagents were maintained at 37˚C during the experiment. Cells were seeded on plates coated with bovine plasma fibronectin (at 1:50 dilution in 1x PBS for 2 hour). Confluent cells were gently washed with 1x PBS and dye containing Lucifer Yellow (2.5 mg/ml) and

Texas Red Dextran (1 mg/ml), dissolved in 1x PBS, was added. Texas Red Dextran was used as a control for the scrape as the molecular weight is too large to pass through a gap junction (3 kDa; data not shown). The cells were scraped with a scalpel and incubated with the dye at room temperature for 5 min. The dye was removed before washing the cells with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ in 1x PBS. The cells were incubated with antibiotic-free DMEM for 5 minutes at 37˚C. 4% PFA was used to fix the cells for 20 minutes at room temperature, followed by DAPI (in 1x TBST) staining for another 20 minutes at room temperature and mounting (with 25% antifade, 75% glycerol) before imaging with a confocal microscope. We measured the total surface area of the dye transferred on both sides of the scrape using ImageJ. During quantification, surface area of dye transferred in HEK^{Par} cells was normalized to 1. Dye transfer in HEK^{BTK} is shown as fraction of dye transfer compared to HEK^{Par}. Statistical data were generated using ImageJ application (1.53t) and GraphPad prism.

Activation of endogenous BTK

Daudi (B lymphocyte isolated from the peripheral blood of a 16-year-old male, Burkitt's lymphoma patient) cells were seeded to reach $1x10^7$ cells/ml density. 10 µg/ml of α-IgM antibody was added to the cells and incubated at 37˚C for 45 min (optimized and modified from [470]). The cells were then lysed prior to Western blot analysis.

Parachute assay

Plates of donor and recipient cells were grown to confluency. The donor plate was incubated with the cell-permeant dyes Dil (#42364, Sigma-Aldrich) and Calcein AM (#14948, Cayman Chemical Company) for 20 min at 5 µM and 1 µM, respectively. Dil is a carbocyanine dye that is confined to the membrane cell. The nonfluorescent Calcein AM

is a cell-permeant dye that is converted to a green fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. Calcein can only pass-through gap junctions [471, 472]. Donor cells (one donor plate was used for both untreated and treated samples for each experiment) were then added to recipient cells at 1:100 dilution and incubated at 37˚C for the indicated time points. After 240 min of incubation, the activating antibody was added, and the cells were further incubated for another 45 minutes. This was followed by confocal imaging to determine if the green fluorescent dye from the donor cells had transferred into the recipient cells. Such transfer is a positive indication of gap junction intercellular communication. At least 10 random microscopic fields per group were photographed and counted, and the experiments were repeated three times. Statistical analysis was done by GraphPad prism. Y-axis units were calculated as number of recipient cells per donor cell.

Confocal imaging

All cell immunofluorescence images were acquired on a Zeiss LSM 800 Confocal system using appropriate numerical aperture objectives and appropriate filter sets.

Statistical analysis

All data were analyzed by using GraphPad Prism 8.0 and presented as the mean ± SD. Statistical analysis performed in GraphPad Prism 8.0 were either one-way ANOVA with a Neuman-Keuls post-hoc analysis or Student's t-test where appropriate; *p*-values <0.05 were considered statistically significant.

12.3 Preliminary results

BTK phosphorylates the Cx43CT domain *in vitro* – Kinase screening

Rat Cx43CT (residues V236-I382) was purified as described [239, 257]. The purified protein was sent to Eurofins Scientific to perform an *in vitro* kinase screening assay. Each kinase had a positive control peptide for comparison. The *in vitro* phosphorylation reaction was incubated at 30˚C for 40 minutes and then transferred to P30 Filtermat for substrate capture. Merck Millipore radiometric assay was used to determine phosphorylation levels. The results showed BTK to be one of the kinases that phosphorylate Cx43CT. [Kinase screening was done with the help of Dr. Hanjun Li].

BTK phosphorylates Tyr residues in Cx43CT – Mass spectrometry

Following the kinase screening, mass spectrometry analysis was done to identify the Cx43CT residues that were being phosphorylated by BTK. An *in vitro* kinase reaction was performed by incubating purified Cx43CT with active BTK overnight at 30˚C. The reaction was stopped with ice and 10 nmol of protein was run on an SDS-PAGE gel followed by Coomassie Blue staining. The Cx43CT bands were excised and sent to the Harvard Center for Mass Spectrometry. The results indicated Y247, Y265, Y267, Y313 to be phosphorylated by BTK (Table 12.1).

Peptide Sequence	Start-end (residue number)	Peptide Mass (Calculated)	Peptide Mass (Actual)	Number of phospho-peptides	Phosphorylation site
SDPyHATTGPLSPSK	244-258	1557.6	1637.72	110	Y247
yAYYNGcSSPTAPLSPMSPPGYK	265-287	2435.7	2588.09	39	Y265
YAyFNGcSSPTAPLSPMSPPGYK	265-287	2435.7	2588.09	12	Y267
YAYFNGcSSPTAPLSPMSPPGyK	265-287	2435.7	2588.09	9	Y286
yAYFNGcSSPTAPLSPMSPPGyK	265-287	2435.7	2668.05	1	Y265/Y286
QASEQNWANySAEQNR	304-319	1895.9	1975.79	80	Y313

y- Phospho (+79.97 mass); c -Carbamidomethyl (+57.02 mass).

Table 12.1

Phosho-Tyr containing peptides of the Cx43CT, *in vitro* phosphorylated by BTK, identified from mass spectroscopy

12.4 Results

Tyr residues of Cx43CT phosphorylated by BTK as seen by an *in vitro* kinase assay

We further wanted to validate the mass spectrometry data by running a Western blot of the *in vitro* kinase reaction sample. There are a total of six tyrosine residues present in the purified Cx43CT: Y247, Y265, Y267, Y286, Y301 and Y313. From this WT Cx43CT, six other constructs were prepared by mutation. Each construct had all the Tyr residues mutated to Phe (due to similarity in structure of Tyr and Phe and the latter lacking the hydroxyl group that is phosphorylated) except one Tyr. These constructs were individually subjected to an *in vitro* kinase reaction by incubating them with active BTK overnight. The overnight reactions were run on polyacrylamide gel followed by Western blot analysis (Figure 12.1). Apart from Y267, all other Tyr show phosphorylation by BTK, although, the intensity of phosphorylation is higher for Y247, Y265, Y313 compared to Y286 and Y301.

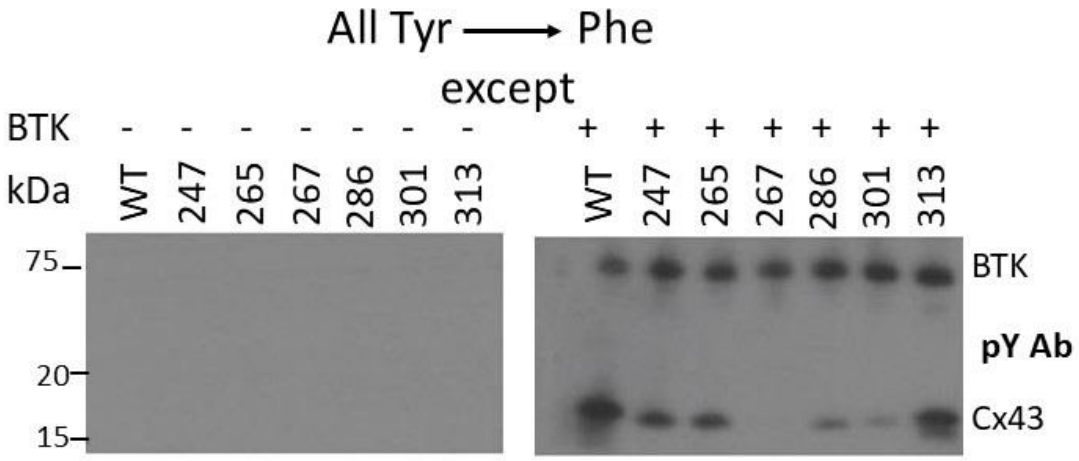


Figure 12.1. Identifying Cx43 Tyr residues phosphorylated by BTK using *in vitro* kinase assay: Purified Cx43CT was incubated with active BTK for the *in vitro* kinase reaction and then subjected to Western blot analysis using general phospho-tyrosine antibody. The lanes on the right of the WT indicate mutated constructs where all Tyr of the Cx43CT were mutated to Phe except the one mentioned on top of the lane. The control blot on the left had no active BTK added. On the right blot, bands of phospho-Cx43 and phospho-BTK can be seen.

Validation of Cx43 pY286 antibody

Among the Tyr residues identified by Western blot to be phosphorylated by BTK, Y286 is a novel site that has not been characterized previously. To validate the pY286 antibody, LA25 cells were used. These cells have been previously used to validate phospho-specific antibodies for Cx43 tyrosines [222]. LA25 cells have a temperature sensitive Src (inactive at 42˚C and active at 37˚C) and both versions were used for validation. Prior to Western blot and immunofluorescence analysis, antibody samples were incubated without and with non-phospho (Np) or phospho (p) peptide. The phospho peptide binds to the antibody and prevents it from binding to pY286 of Cx43. Data showed phosphorylation of Y286 with and without non-phospho peptide. However, no phosphorylation signal was noticed after blocking with phospho-peptide. Immunofluorescence data also showed similar results, indicating specificity of the antibody (Figure 12.2 A and 12.2 B).

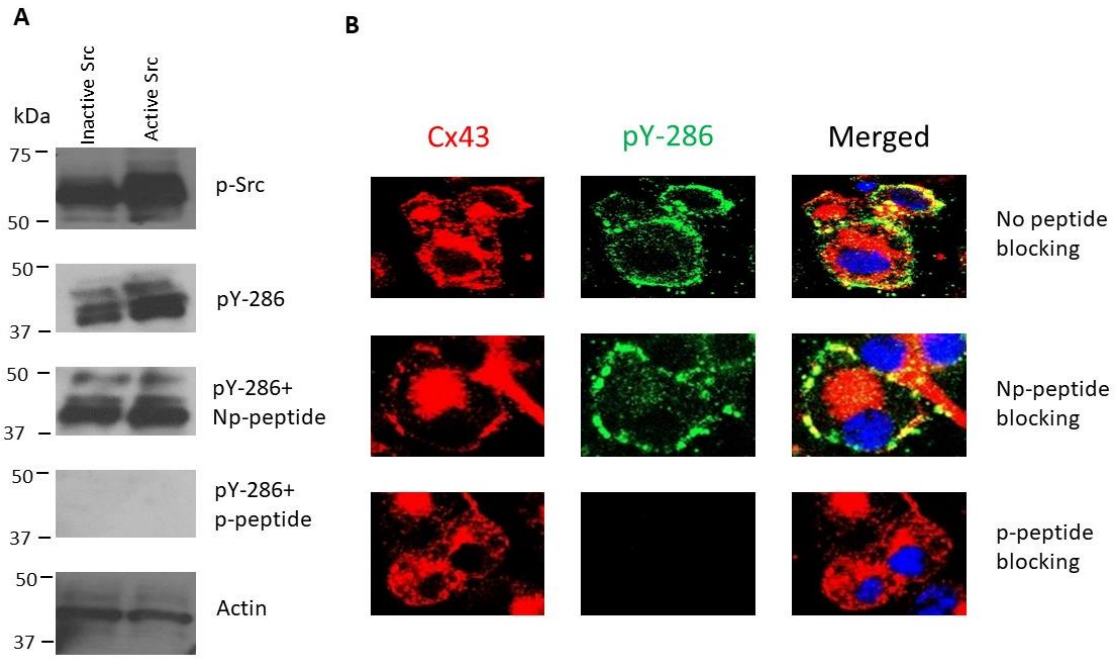
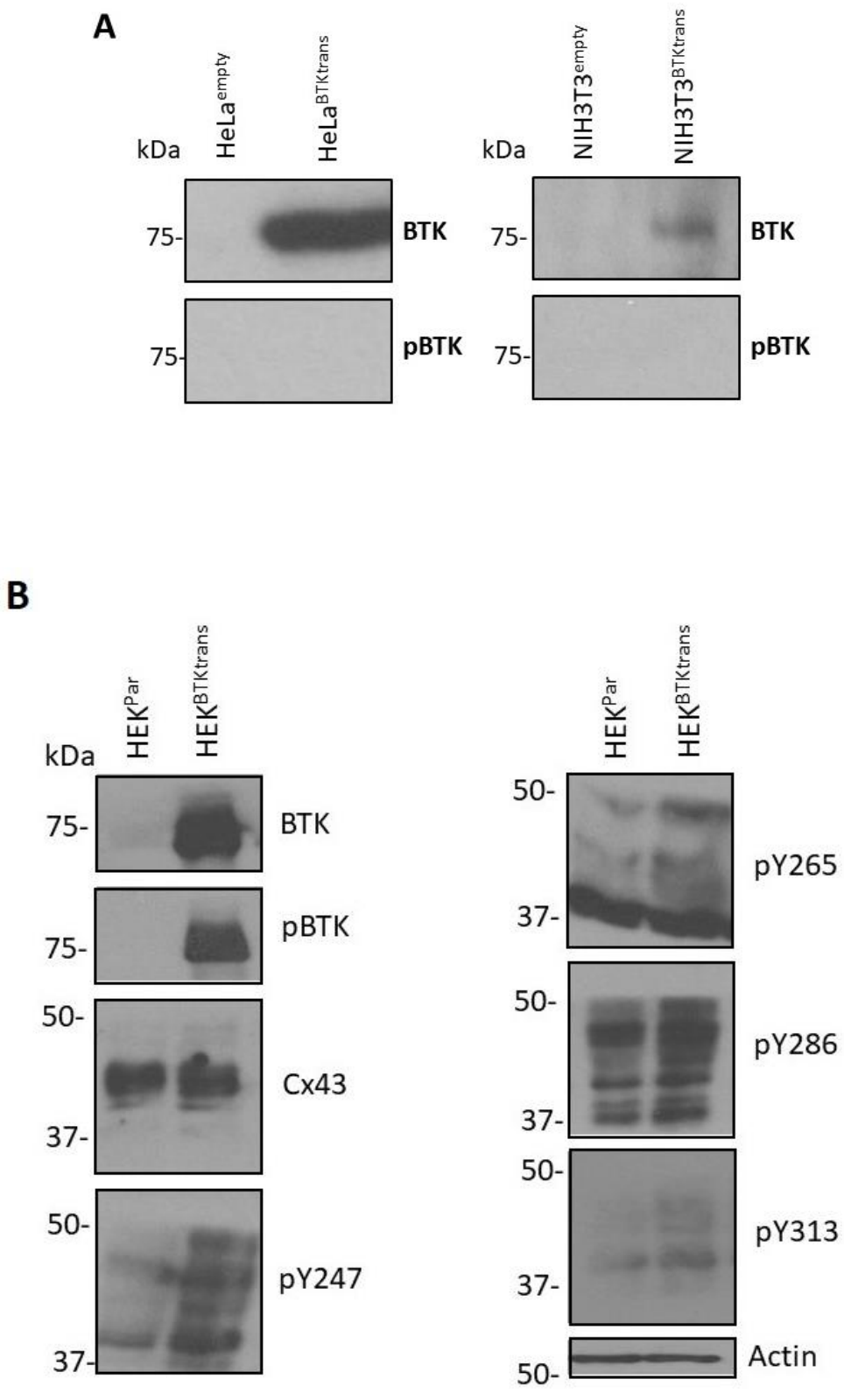


Figure 12.2. Validating the Cx43 pY286 antibody: LA25 cells were used to validate Cx43 pY286 antibody. A: On the left panel (Western blot), the antibody was incubated with without Non phospho-peptide (Np-peptide) or phospho peptide (p-peptide) and compared to the non-incubated control. B: Immunofluorescence data shows the same result of incubation of antibody with or without Np or p-peptide. Red: Cx43, Green: pY286, Blue: DAPI, Yellow: colocalization.

Phosphorylation of Cx43 Y247 increases in the presence of BTK

HeLa cells do not express Cx43 endogenously. This can be used to our advantage to study Cx43 as we can express Cx43 in different forms (WT or mutated) as and when necessary. In this study, HeLa cells stably expressing Cx43, were transiently transfected with BTK, incubated overnight after transfection, followed by lysis for Western blot analysis (Figure 12.3 A). BTK is activated by Tyr phosphorylation of its kinase domain. When the transfected HeLa samples were blotted with phospho-BTK (pBTK) antibody, no signal was noticed. This was due to the lack of kinases that activate BTK including Lyn. Thus, the focus was shifted to using cell lines that are known to have the kinases that activate BTK. Two such cell lines were chosen (that do not express endogenous BTK) – NIH3T3 and HEK293T (HEK^{Par}). Both these cell lines were transiently transfected with BTK and analyzed by Western blot. NIH3T3 did not show activation of BTK (Figure 12.3 A), while HEK293T did (Figure 12.3 B). Thus, HEK cells were used for further experimentation. Western blot data showed increase in phosphorylation of Y247. Consequently, a cell line was generated for HEK^{Par} cells that stably expressed BTK (HEK^{BTK}). In HEK^{BTK}, the increase in the phosphorylation of Y247 was statistically significant. When HEK^{BTK} samples were blotted with Cx43 antibody, there was decrease in the P2 isoform along with increased P0 levels (Figure 12.3 C). Thus, overexpression of BTK affects the migration pattern of Cx43 and increases phosphorylation of Y247 in HEK293T cells.


A
kDa
HeLa^empty
HeLa^BTKtrans
75-
BTK
75-
pBTK
kDa
NIH3T3^empty
NIH3T3^BTKtrans
75-
BTK
75-
pBTK
B
kDa
HEK^Par
HEK^BTKtrans
75-
BTK
75-
pBTK
50-
Cx43
37-
50-
pY247
37-
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pY313
37-
50-
Actin

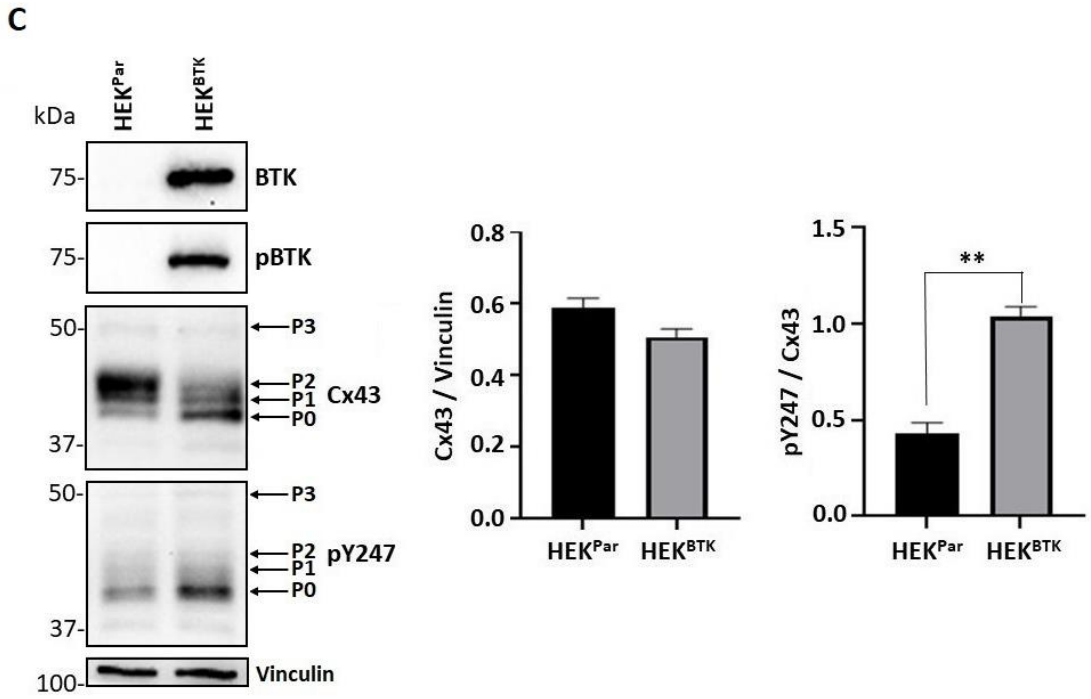


Figure 12.3. Effect of BTK on phosphorylation of Cx43 Tyr residues**:** Panel A: left picture shows BTK and pBTK levels in HeLa cells transiently transfected with empty vector ($HeLa^{empty}$) and BTK plasmid ($HeLa^{BTKtrans}$); right picture shows BTK and pBTK levels in NIH3T3 cells transiently transfected with empty vector ($NIH3T3^{empty}$) and BTK ($NIH3T3^{BTKtrans}$). Panel B indicates the Tyr phosphorylation of Cx43 by BTK in HEK^{Par} and HEK^{Par} cells transiently transfected with BTK ($HEK^{BTKtrans}$) using Western blot analysis. Panel C shows the same data in HEK^{Par} and HEK^{BTK} stable cells. Data was quantified with ImageJ and GraphPad prism with the help of Student's *t*-test (n=3, $^{**}p<0.01$).

BTK decreases Cx43 plaque size

When active BTK phosphorylates its substrates, it is present at the plasma membrane. To see its colocalization with Cx43, immunofluorescence was done. HEK^{Par} and HEK^{BTK} samples were stained with both Cx43 and BTK or phospho-BTK antibodies. Localization of Cx43 in HEK^{Par} cells was at the plasma membrane with little to none found intracellularly (Figure 12.4 left panel). In the HEK^{BTK} samples stained with Cx43 and BTK, it was hard to determine colocalization as BTK signal was present throughout the cell cytoplasm. In the stable cells stained with Cx43 and phospho-BTK, few regions were noticed to indicate colocalization. One noticeable difference between the parental and stable cell lines was the size of the Cx43 plaques at the membrane. The size of the plaque was significantly decreased in the presence of the kinase (Figure 12.4 right panel).

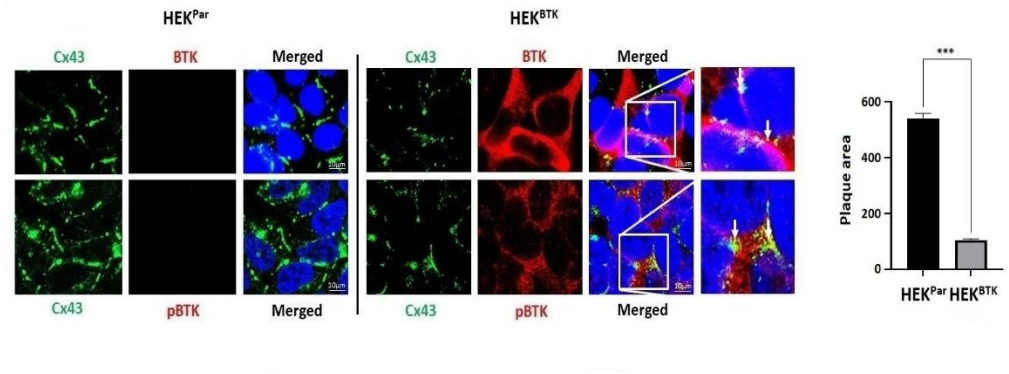


Figure 12.4. Studying effect of BTK on localization of Cx43 in HEK293T cells**:** Immunofluorescence was used to visualize Cx43 in HEK^{Par} and HEK^{BTK} cells. Green: Cx43, Red: BTK or phospho-BTK, Blue: DAPI. White arrows point to colocalization pockets of Cx43 with either BTK or pBTK. Cx43 plaque area was quantified with ImageJ and GraphPad prism. (n=10, ***p<0.001).

BTK decreases Cx43 levels that are present within the gap junction plaque

Gap junctional plaques are resistant to mild-detergent solubilization. Following Triton-X 100 extraction, ultracentrifugation separates the gap-junctional Cx43 (insoluble fraction) from the non-gap junctional Cx43 (soluble fraction). The assay result showed that soluble fraction was increased, and insoluble fraction was decreased in the HEK^{BTK} samples compared to that in the control (Figure 12.5).

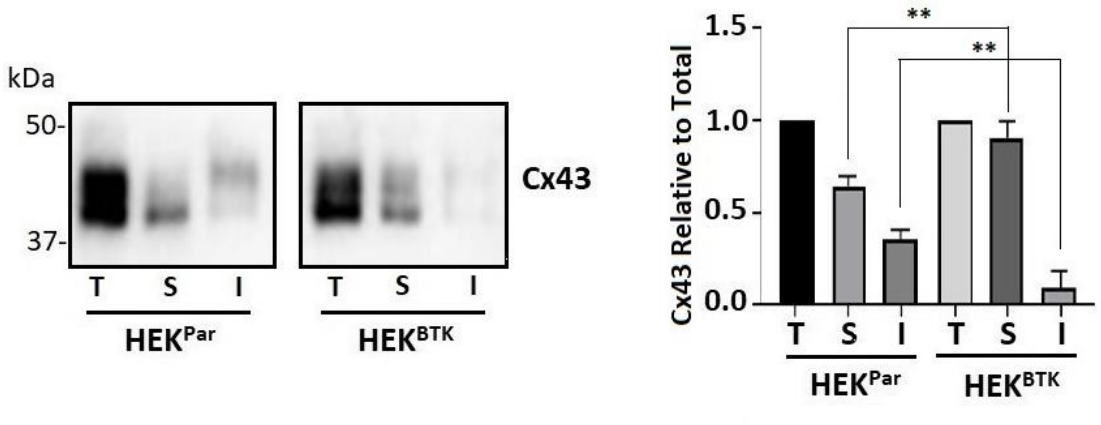


Figure 12.5. Effect of BTK on stability of Cx43 at the gap junction plaque: The mild detergent Triton X-100 was used to extract Cx43. Gap junctional fraction of Cx43 is referred to as insoluble (I), non-gap junctional fraction is soluble (S), total sample (T) includes insoluble (I) + soluble (S) fractions. Statistical analysis generated by ImageJ and GraphPad prism (n=3, **p<0.01).

BTK decreases GJIC in HEK293T cells

Lucifer Yellow (444 Da) is an anionic dye that can pass through Cx43 gap junction channels. With increase in GJIC, there is increase in the area of this dye spread through the channels. Scrape loading dye transfer assay was used to assess GJIC in HEK293T cells. The data shows a marked decrease in the dye transfer area in HEK^{BTK} samples (Figure 12.6). Less dye transfer indicates less GJIC. Unlike Lucifer Yellow, Texas Red Dextran has a higher molecular weight (3 kDa) which renders it gap junction impermeable and staining with this is used as a control to assess the data more effectively.

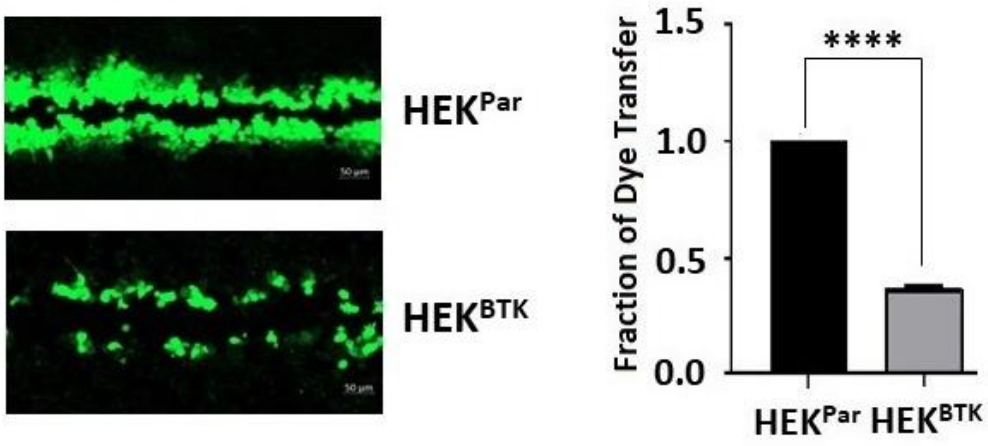


Figure 12.6. Effect of BTK on GJIC in HEK cells: Scrape loading dye transfer assay was used to study gap junction mediated intercellular communication (GJIC) in HEK^{Par} and HEK^{BTK} cells. Green: Lucifer Yellow. Area of dye transferred was measured by ImageJ and data was quantified by GraphPad prism (n=3, ****p<0.0001).

Activation of BTK increases Tyr phosphorylation of Cx43 in B lymphocytes

After using HEK cells to study the role of BTK in phosphorylation of Cx43, Daudi cells (B lymphocytes) were used to determine if the results were consistent in a cell line that endogenously expresses BTK. Endogenous BTK was activated in Daudi cells using α-IgM antibody. Activation of BTK was significantly increased. It corresponded with an increase in the pY247 levels along with that of the P2, P3 and P4 bands compared to the non-activated sample (Figure 12.7). The migration pattern of Cx43 was unchanged compared to samples with non-activated BTK. The increase in the pY247 is consistent with that in HEK cells.

In the immunofluorescence data, there is a lower phospho-BTK level in the non-activated cells. BTK activation also shows the presence of few colocalization pockets of Cx43 and active BTK (Figure 12.8).

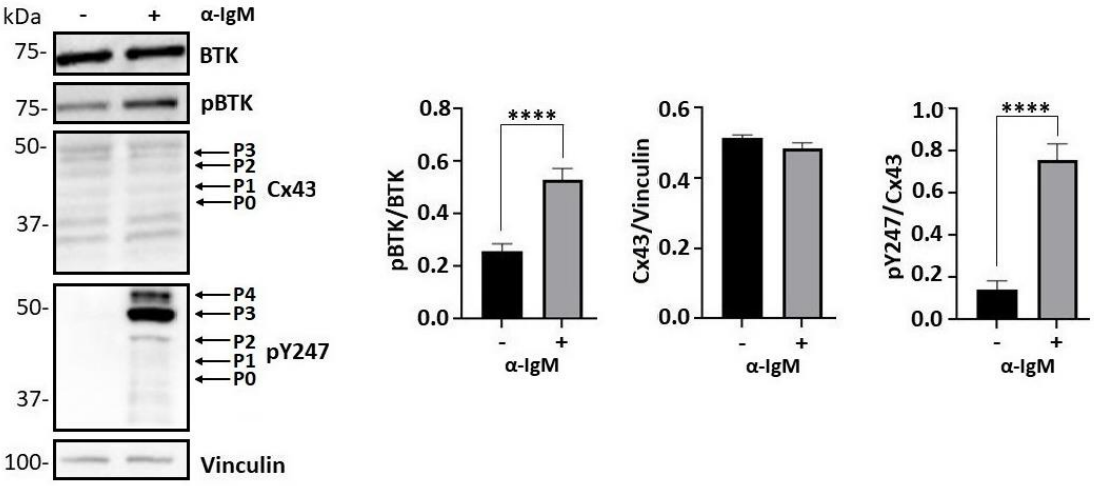


Figure 12.7. Effect of BTK on Cx43 phosphorylation in T lymphocytes: Daudi cells were used for Western blot analysis of Cx43 phosphorylation as a result of BTK activation through the B-cell receptors. Arrows point to the different migrating isoforms of Cx43. Band signals were measured by ImageJ, followed by quantification with GraphPad prism (n=3, ****p<0.0001).

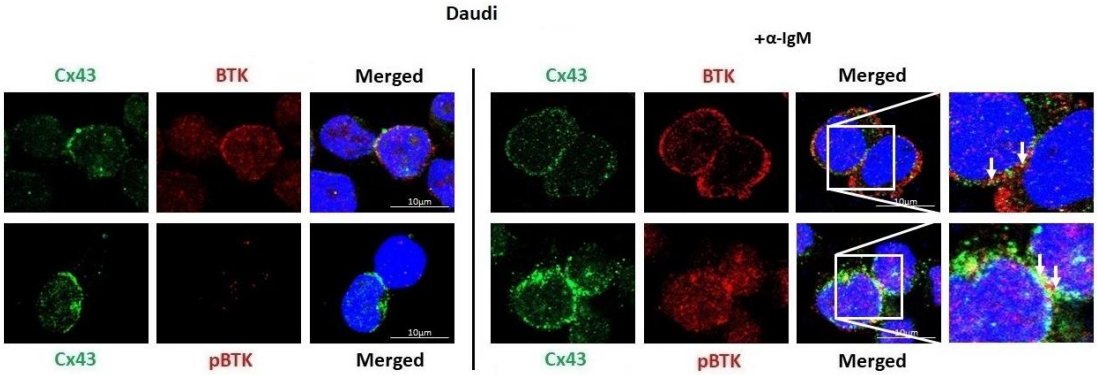


Figure 12.8. Effect of active BTK on cellular localization of Cx43 in B lymphocytes: Immunofluorescence data was used to visualize localization of endogenous Cx43 in Daudi cells with and without activation of BTK. Green: Cx43, Red: BTK or pBTK, Blue: DAPI. White arrows indicate pockets of colocalization of Cx43 with BTK or pBTK.

Activation of BTK decreases GJIC

As Daudi cells are non-adherent cells, parachute assay technique was used instead of scrape loading dye transfer assay to assess GJIC. For each replicate of the experiments, there was one donor plate (cells loaded with both DIL and Calcein-AM) and two recipient plates (unloaded cells for non-activated and activated). Few cells from the donor plate were dropped onto both recipient plates and incubated for the indicated time points. During this time, the calcein (green) from the donor cells is transferred to the recipient cells only through gap junctions. A higher number of green recipient cells around one donor cell indicates increased dye transfer, which in turn indicates more GJIC. Activation of BTK causes significant decrease in the number of recipient cells surrounding one donor cell, thus indicting decrease in GJIC (Figure 12.9).

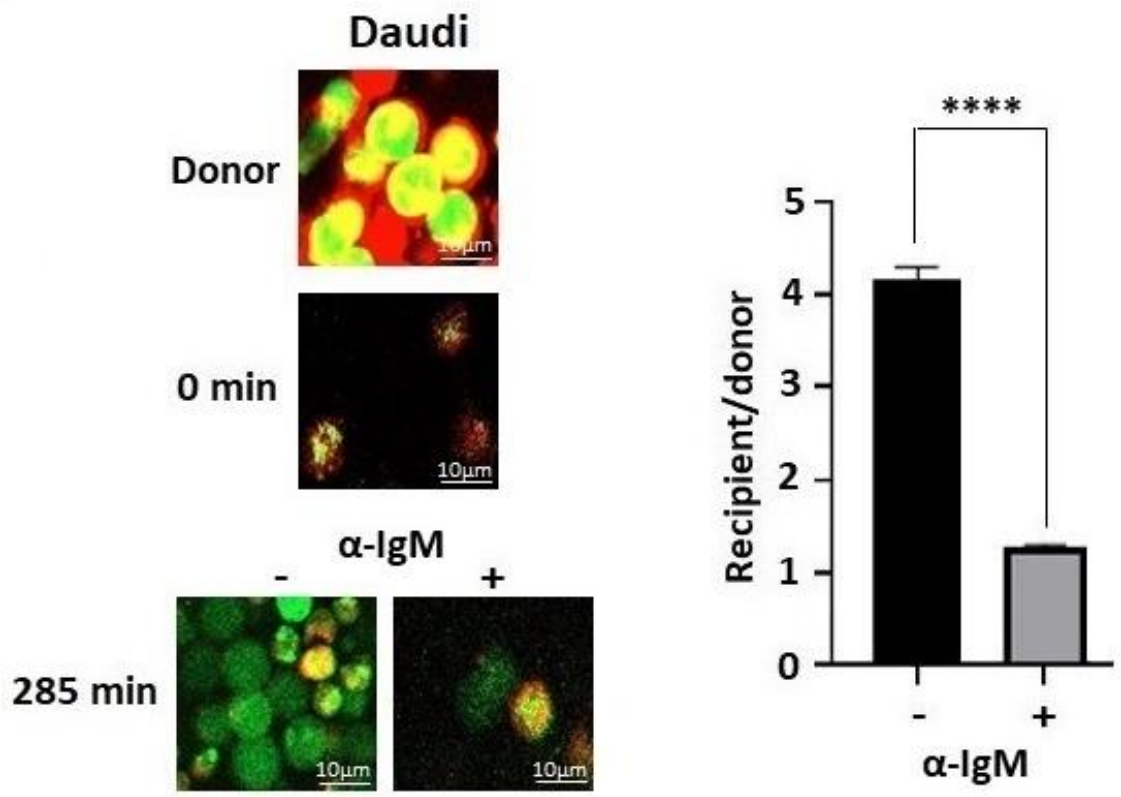


Figure 12.9. Effect of active BTK on GJIC in B lymphocytes**:** Parachute dye coupling assay was used to determine GJIC in Daudi cells with and without BTK activation. Red/Yellow: Donor cells, Green: Recipient cells that have taken up the dye calcein from the donor cells. Statistical analysis was done using GraphPad prism (n=3, ****p<0.0001).

12.5 Discussion

Cx43 has the most ubiquitous expression in cells of hematopoietic lineage [301]. Also, Cx43 has the highest expression in B cells compared to other connexin isoforms [301, 305]. Gap junctions in B cells play major roles in their normal functioning including B cell motility, migration and adhesion to epithelial cells [312, 466, 473]. Presence of Cx43 has been indicated between two B cells as well as at the immunological synapse between B cells and other immune cells [474]. Phosphorylation of Cx43 residues Y247 and Y265 is required for B cell spreading [475]. Mutation of these residues affect the B cell spreading in response to receptor mediated signaling [466, 475].

Cell-cell communication among naive B cells via methods including gap junctions, is necessary for various reasons. Naïve B cells can communicate with each other during their formation in the bone marrow [312]. They need to communicate in bone marrow and spleen during maturation [468, 476]. Naïve B cells interact with dendritic cells before and during antigenic transfer [477]. Besides being present among tissues in organs, there are instances where B cells need to migrate from niche to niche. After their successful production, they need to migrate from bone marrow to spleen for the final steps of maturation [478]. After activation of naïve B cells, they need to migrate rapidly (guided by chemokines) to target organs to mediate humoral immune response [479]. Evidences show relation between Cx43 and regulation of cell migration. Relative migration of glioma cells is increased as a result of reduction in Cx43 expression and this occurs by affecting the direction of migration and increasing the cell speed [480]. A study showed that expression of Cx43 in HeLa cells reduced cell motility due to increased adhesiveness [481]. Post migration to their target organs, B cells interact with other cells through gap junctions.

Cx43 gap junctions are formed at B cell synapses and between B cells and T cells and participate in adaptive immune response [482]. Cx43 mediated GJIC is involved in transcytosis of IgA in B cells [483]. Thus, there is a need for both high and low levels of GJIC in B cells depending on the phase of the B cell mediated adaptive immune response. In this study, we noticed a decrease in GJIC after activation of BTK. Activation of BTK occurs during B cell activation and the resulting decrease in GJIC can enhance motility of B cells leading to increased migration from one site to another. This may promote the adaptive immune response mediated by B cells in their target organs.

Many different scaffolding proteins are involved in regulating stability of Cx43 at the plaque. As discussed earlier, Drebrin and ZO-1 are two such proteins. However, ZO-1 is not expressed in Daudi cells. The role of this protein is probably substituted by other scaffolding proteins in the immune cells lacking it. Additionally, there is no evidence in literature to suggest the presence of ZO-1 in the immunological synapse unlike ZO-2 [484]. The latter protein may be a substitute for the former in the lymphocytes. In HEK cells, we observed a differential migratory pattern of Cx43 in cells with and without BTK. There is an increase in the P0 isoform of Cx43 with activation of BTK. On the other hand, the P2 isoform decreases. As mentioned in [221, 239], the P2 is associated with connexin in the gap junction plaque and is involved in GJIC. Decrease of P2 in this study indicates decrease in gap junctional Cx43 upon BTK activation. Subsequently, increase of the P0 and P1 isoforms indicates increase in non-gap junction forming Cx43. Also, the overall phosphorylation level of Tyr 247 of Cx43 shows a significant increase following BTK activation. Consistent with the data of total Cx43, the P0 isoform level when blotted with phospho-specific Y247 also shows an increase. These data suggest the instability of Cx43

at the gap junction which is causing it to transform to the non-gap junction region from gap junctional plaques. The activation of many kinases including protein kinase A, cyclin B kinase, casein kinase 1, protein kinase C, MAPK, can cause phosphorylation of Ser and Tyr residues of Cx43 leading to instability of the connexin at the plaque and can affect GJIC [165, 209, 254, 280, 485]. Our TX-100 solubility assay data is also consistent with this observation as we noticed an increase in the soluble (non-gap junction) fraction of Cx43 along with a decrease in the insoluble (gap junction plaque) fraction in the presence of BTK. This also suggests that Cx43 stability at the plaque decreases after BTK activation.

The assembly of connexins into plaques and disassembly from the plaques have been extensively linked to the action of various kinases on the connexins [259, 486]. A 'kinase program' is mentioned, which states that Cx43 is sequentially phosphorylated leading to its disruption from the gap junction plaque and plasma membrane [259]. Recent evidence suggests the role of kinases and cytoskeletal elements (such as Z0-1 [268]) in regulating the size of the gap junction plaque and gap junction disassembly [7]. Drebrin is another cytoskeletal element linked to regulation of plaque size [487]. In this study, we noticed a decrease in the gap junctional plaque size as seen in the immunofluorescence data in HEK. This also suggests a decrease in the stability of Cx43 at the plaque, similar to the result observed by the differential migration pattern of Cx43 in our Western blot data. However, plaque size did not seem to change due to BTK activation in Daudi cells. The absence of ZO-1 and Drebrin in B cells may be one contributing factor for the lack of change in plaque size. Our immunofluorescence data in both HEK cells and Daudi show little to no change in localization of Cx43, although there seems to be slight increase in internalized Cx43 in Daudi cells upon BTK activation. Besides phosphorylation by a kinase

at a Tyr site, a combination of other factors is necessary for internalization of connexins such as sequential phosphorylation of various Tyr and Ser residues and dissociation from cytoskeletal elements (including Drebrin, ZO-1, tubulin) [222, 259, 488]. The cytoplasmic CT domain of Cx43 has Ser, Thr, Tyr residues whose phosphorylation can regulate channel gating [489]. Phosphorylation also changes the net charge of the carboxyl group of connexin residues. This change can affect the binding affinity of protein partners towards the CT domain. The change in net charge can also regulate the kinetics of pH or voltage regulated gating [490]. Cx43 channels occur in more than one conductive state. Phosphorylation can alter the conductive state of the Cx43 channels as seen in case of phosphorylation by PKC. When unitary junctional conductance was recorded in SKHep1 cells transfected with Cx43, channel populations with two conductance values were revealed: 60 pS and 90 pS. Following treatment with a PKA activating molecule, the 60/90 ratio was increased five-fold [491]. Phosphorylation can also cause the Cx43 channels to transform into a non-conductive state as seen after phosphorylation by Src [290]. This study showed that Y247 and Y265 phosphorylation by v-Src reduced the conductance by almost 80%. In both the cell lines tested, we noticed marked decrease in gap junctional intercellular communication (GJIC). This decrease in GJIC without a strong increase in internalization of Cx43 suggests that BTK is affecting channel gating, causing channel closure. Closure of channels and decreased conductance may enable the B cells to respond to chemokine receptors by becoming more motile so that they can reach tissues to interact with other immune cells or even proliferate and differentiate into memory B cells.

CHAPTER THREE

Role of interleukin 2-inducible T-cell kinase (ITK) in regulating Cx43 gap junctional intercellular communication

The bulk of data in this chapter has been published in Basu et al.

Biomolecules, 2023 [464]

13.1 Introduction

Gap junctions are channels connecting two cells that allow ions and small molecules to pass through them as described earlier [1, 2, 21-26]. Gap junctional intercellular communication (GJIC) is necessary for maintaining the cellular network and cooperativity among cells in a system [492]. Normal electrical conductance in cardiomyocytes is dependent on GJIC [493]. Gap junction based electrical synapses are important for regulating the development and function of the nervous system [494]. GJIC in osteocytes maintains the levels of calcium and is thus important for bone formation and bone resorption [495]. GJIC between lung macrophages and epithelial cells is necessary to maintain lung homeostasis [496]. GJIC maintains the electrical coupling between pancreatic beta cells and delta cells [497]. It is also a key player in immune responses in our body [301].

As mentioned earlier, Cx43 is the most ubiquitous connexin expressed in human tissues [465]. It is also the predominant gap junction protein in the immune cells [301, 498]. It is involved in various T cell mediated processes. Cx43 is necessary for the maturation of T lymphocytes in the thymus [498], for contact of T cells with other T cells or with other immune cells while residing in the lymph nodes [305, 499], for transfer of antigenic peptides from antigen presenting cells to T cells during antigen presentation [482, 499], and for transmigration of T cells across the endothelial cell layer while migrating through the vascular vessels [364]. Thus, Cx43 plays a role in many physiological processes involving the T cells.

Recently, a kinase screening was done with purified Cx43CT being screened for phosphorylation by different kinases. Interleukin 2-inducible T-cell kinase (ITK, involved

in T cell activation) was one of the kinases that was shown to phosphorylate the CT of Cx43. Following this, a mass spectrometry analysis was done, which revealed five Tyr residues to be phosphorylated by ITK. Based on these results, we studied the effect of ITK on GJIC through phosphorylation of the Cx43CT.

13.2 Materials and methods

Antibodies and detection reagents

α-Vinculin (#4650S, Western blot dilution [WB] - 1:1000), α-ITK (#2380S, WB - 1:1000, IMF - 1:200), α-rabbit-Alexa 488 (#4413, IMF - 1:500), α-mouse-Alexa 647 (#4410, IMF - 1:500), anti-rabbit IgG (#7074S, WB - 1:5000), anti-mouse IgG (#7076S, WB - 1:5000) were purchased from Cell Signaling; α-phospho-ITK (#PA5-40292, WB - 1:1000, IMF - 1:100) was purchased from Invitrogen; α-Cx43 (#C6219, WB - 1:2000, IMF - 1:500); Cx43 pY247 antibody was custom made from LifeTein and α-Actin (#A5441, WB - 1:5000) were purchased from sigma; Lucifer Yellow (#L453, 2.5 mg/ml, was purchased from Life Technologies); and DAPI (#5748) was purchased from Tocris. Additionally, α-CD3 (clone OKT3) and α-CD28 antibodies were purchased from BioLegend (#317317) and Invitrogen (#16-0289-81), respectively.

Cell culture

HeLa cells (gift from Dr. Steve Caplan, University of Nebraska Medical Center) and HEK293T cells (gift from Dr. Myron Toews, University of Nebraska Medical Center) were cultured in Dulbecco's modified Eagle medium or DMEM (from Corning) in a 37˚C incubator with a humidified 5% CO_2 atmosphere. The DMEM was supplemented with 2mM L-glutamine (HyClone), 0.2% Normocin (Invitrogen), 1% Penicillin-Streptomycin (from Corning) and 10% fetal bovine serum. Jurkat cells (#T1B-152 from ATCC) were

cultured in Roswell Park Memorial Institute Medium (RPMI) 1640. RPMI 1640 powder (ThermoFisher) at 0.84% was added to water and this solution was further prepared to contain 25 mM D-Glucose, 17.85 mM Sodium Bicarbonate, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM Sodium Pyruvate, 10% fetal bovine serum and 1% Penicillin-Streptomycin. The pH of this medium was 7.4.

Transient transfection

HEK293T cells were cultured in DMEM. Upon reaching 75% confluency, these cells were transfected with the plasmids of interest (at 2.5µg/ml) was done using X-tremeGENE and OptiMEM. They were maintained for approximately 16 hours prior to lysis for Western blot or fixing for immunofluorescence.

Stable cell line generation

HEK293T cells (HEK or HEK^{Par}) were cultured in DMEM up to 60% confluency. These cells were transfected with ITK plasmid (at 2.5 µg/ml) under antibiotic free condition and cultured for another 72 hours. The media was replaced with selection media which contained 2 µg/ml puromycin. Cells were further grown until monoclonal cell lines were obtained, which was achieved by preparing cloning disks made from Whatman paper. Clones were finally screened and selected by comparing Western blot and immunofluorescence data (referred to here as HEK^{ITK}).

Cell lysis for Western blot

Cultured cells were rinsed with 1x Tris-Buffered Saline (TBS) prior to lysis. Lysis buffer was added to HeLa and HEK cells, followed by scraping these cells from the plate with a cell scraper. The scraped cells were transferred to a centrifuge tube and kept on ice

for 30 minutes after subjecting them to sonication for 10 seconds. Jurkat cells were pelleted down (at 125 *x g*, 8 minutes) after rinsing with 1x TBS. The pellet was resuspended in lysis buffer and kept on ice for 30 minutes. The lysis buffer contained protease/phosphatase inhibitor buffer, 2 mM Phenylmethylsulfonyl fluoride (PMSF), 2 μM pepstatin A. The cell suspensions kept on ice were centrifuged at 1500 RPM for 10 minutes at 4˚C and the lysates (the supernatant) were removed from the pellet and kept on ice. These lysates were quantified by bicinchoninic acid assay (BCA) and normalized to a final concentration of 2 mg/ml protein.

Western blot

Confluent cells were washed in 1x TBS and lysed in a buffer containing protease and phosphatase inhibitors, 2 mM PMSF, 2 mM Pepstatin A, and 1% Triton X-100. Bicinchoninic acid assay (BCA) was used to measure the concentration of lysates. 6x loading dye (69% 4x Tris-Cl/SDS, 29% glycerol, 0.3 M SDS, 0.6 M DTT, 0.19 mM bromophenol blue, dissolved in water, pH 6.8) was added to the lysate and 30 μg of protein was loaded on a 10% SDS-PAGE gel, followed by transfer to PVDF (Millipore) membrane. Blocking of membrane was done in 5% bovine serum albumin (BSA)/TBS with tween-20 for 1 hour (you spell out minutes so spell all these out) before primary antibody incubation overnight. The membrane was washed in TBS with tween-20 before secondary antibody incubation for 1 hour. Signal was detected with SuperSignal West Femto (Thermo Scientific) substrate using digital imager (iBright, Thermo Fisher Scientific). Quantification is the result of measuring the sum of intensity of all bands shown in the blot.

Immunofluorescence

Cover slips (Neuvitro) were coated with fibronectin (solution made in 1x PBS at 1:50 ratio) and HEK cells were seeded on them overnight to reach 98% confluency. For the Jurkat cells, PLL coated cover slips were used. The cover slips were transferred to 24 well plates. (1/well) and rinsed with 1x PBS. 300 µl of fixing solution (3.7% formaldehyde, 0.3% TX-100, 1x PBS) was added to each cover slip and incubated at room temperature for 30 minutes. They were rinsed with 1x PBS twice for 10 minutes each time and incubated with immunofluorescence blocking buffer (1% bovine serum albumin (BSA), 0.3% TX-100, 1x PBS) at room temperature for 1 hour. Primary antibodies were prepared in immunofluorescence blocking buffer and added to the blocked cover slips overnight at 4˚C. Next day, the excess primary antibody was removed, and cover slips washed with 1x PBS twice for 10 minutes each time. This was followed by incubation with secondary antibodies (dissolved in immunofluorescence blocking buffer) at room temperature for 1 hour and washing with 1x PBS for 10 minutes. DAPI (4',6-diamidino-2-phenylindole) in 1x PBS was added and incubated for 10 minutes at room temperature, followed by rinsing with 1x PBS for 10 minutes. Cover slips were mounted on glass slides with SlowFade antifade. Excess antifade was blotted off and cover slips were sealed and dried before being imaged with confocal microscopy.

Triton X-100 solubility assay for Western blot

Cultured cells (in 10 cm plates) were rinsed with 1x TBS. 1 ml lysis buffer (protease inhibitor cocktail, 2 mM PMSF, 2 µM pepstatin A) was added to the cells. The cells were scraped off with a cell scraper and transferred to a 2 ml centrifuge tube. Lysis was sone by sonication for 10 seconds and lysates were quantified by BCA method. Following

quantification, samples were normalized to 1.5 mg protein in 1ml solution. 10% TX-100 was added (final concentration-1%) and sample was incubated at 4˚C for 1 hour on rotator. Samples were then vortexed prior to separating them for fractionation. From the 1ml sample, 450 µl was separated and mixed with loading dye and reserved as total protein. Another 450 µl was transferred to ultracentrifuge tubes and fractionated by ultracentrifugation at 100,000 *x g* for 1 hour at 4˚C. Supernatant was carefully removed, mixed with loading dye and reserved as soluble fraction. 450µl of solubilization buffer (8 M urea, 2.5% SDS, 0.1 M dithriothreitol (DTT), protease inhibitor cocktail, 2 mM PMSF, 2 µM pepstatin A) was added to resuspend the pellet, then mixed with loading dye and reserved as insoluble fraction. The fractions were kept at 4˚C overnight prior to Western blot analysis.

***in situ* TX-100 assay**

Cultured cells were seeded on cover slips up to 98% confluency and washed with 1xPBS. Cells were incubated with cold TX-100 buffer (1x PBS, 1% TX-100, protease/phosphatase inhibitor, 1 mM $CaCl_2$, 1 mM $MgCl_2$) at 4˚C for 30 minutes. Following incubation, cells were washed with 1xPBS twice, then fixed and immunostained.

Scrape loading dye transfer assay

Scrape loading of HEK-293T cells was done as described previously [416]. All reagents were maintained at 37˚C during the experiment. Cells were seeded on plates coated with bovine plasma fibronectin (at 1:50 dilution in 1x PBS for 2 hour). Confluent cells were gently washed with 1x PBS and dye containing Lucifer Yellow (2.5 mg/ml) and

Texas Red Dextran (1 mg/ml), dissolved in 1x PBS, was added. Texas Red Dextran was used as a control for the scrape as the molecular weight is too large to pass through a gap junction (3 kDa; data not shown). The cells were scraped with a scalpel and incubated with the dye at room temperature for 5 min. The dye was removed before washing the cells with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ in 1x PBS. The cells were incubated with antibiotic-free DMEM for 5 minutes at 37˚C. 4% PFA was used to fix the cells for 20 minutes at room temperature, followed by DAPI (in 1x TBST) staining for another 20 minutes at room temperature and mounting (with 25% antifade, 75% glycerol) before imaging with a confocal microscope. We measured the total surface area of the dye transferred on both sides of the scrape using ImageJ. During quantification, surface area of dye transferred in HEK^{Par} cells was normalized to 1. Dye transfer in HEK^{ITK} is shown as fraction of dye transfer compared to HEK^{Par}. Statistical data were generated using ImageJ application (1.53t) and GraphPad prism.

Activation of endogenous ITK

Jurkat (T lymphocyte cell line established from the peripheral blood of a 14-year-old, male, acute T-cell leukemia patient) cells were seeded to until $1x10^7$ cells/ml density. 3 µg/ml of α-CD 3 antibody was added to the plates, followed by addition of α-CD 28 antibody (5 µg/ml), which was incubated for 2 hour at 37˚C before lysing the cells for Western blot (optimized and modified from [500, 501]).

Parachute assay

Plates of donor and recipient cells were grown to confluency. The donor plate was incubated with the cell-permeant dyes Dil (#42364, Sigma-Aldrich) and calcein AM (#14948, Cayman Chemical Company) for 20 min at 5 µM and 1 µM, respectively. Dil is

a carbocyanine dye that is confined to the membrane cell. The nonfluorescent calcein AM is a cell-permeant dye that is converted to a green fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. Calcein can only pass-through gap junctions [419, 420]. Donor cells (one donor plate was used for both untreated and treated samples for each experiment) were then added to recipient cells at 1:100 dilution and incubated at 37˚C for the indicated time points. After 240 min of incubation, the activating antibodies were added, and the cells were further incubated for another two hours. This was followed by confocal imaging to determine if the green fluorescent dye from the donor cells is transferred into the recipient cells. Such transfer is a positive indication of gap junction intercellular communication. At least 10 random microscopic fields per group were photographed and counted, and the experiments were repeated three times. Statistical analysis was done by GraphPad prism. Y-axis units were calculated as number of recipient cells per donor cell.

Confocal imaging

All cell immunofluorescence images were acquired on a Zeiss LSM 800 Confocal system using appropriate numerical aperture objectives and appropriate filter sets.

Statistical analysis

All data were analyzed by using GraphPad Prism 8.0 and presented as the mean ± SD. Statistical analysis performed in GraphPad Prism 8.0 were either one-way ANOVA with a Neuman-Keuls post-hoc analysis or Student's *t*-test where appropriate; *p*-values <0.05 were considered statistically significant.

13.3 Preliminary results

ITK phosphorylates the Cx43CT domain *in vitro* - kinase screening

Rat Cx43CT (residues V236-I382) was purified as described [239, 257]. The purified protein was sent to Eurofins Scientific to perform an *in vitro* kinase screening assay. Each kinase had a positive control peptide for comparison. The *in vitro* phosphorylation reaction was incubated at 30˚C for 40 minutes and then transferred to P30 Filtermat for substrate capture. Merck Millipore radiometric assay was used to determine phosphorylation levels. The results showed that ITK was one of the kinases to phosphorylate Cx43CT. [Kinase screening was done with the help of Dr. Hanjun Li].

ITK phosphorylates Tyr residues in Cx43CT - mass spectrometry analysis

Following the kinase screening, mass spectrometry analysis was done to identify the Cx43CT residues that were being phosphorylated by ITK. An *in vitro* kinase reaction was performed by incubating purified Cx43CT with active ITK overnight at 30˚C. The reaction was stopped with ice and 10 nmol of protein was run on an SDS-PAGE gel followed by Coomassie Blue staining. The Cx43CT bands were excised and sent to the Harvard Center for Mass Spectrometry. The results indicated Y247, Y265, Y267, Y286, Y313 to be phosphorylated by ITK (Table 13.1). [Mass spectrometry was done with the help of Dr. Andrew Trease].

Peptide Sequence	Start-end (residue number)	Peptide Mass (Calculated)	Peptide Mass (Actual)	Number of phospho-peptides	Phosphorylation site
SDPyHATTGPLSPSK	244-258	1557.6	1637.72	50	Y247
yAYYNGcSSPTAPLSPMSPPGYK	265-287	2435.7	2588.09	33	Y265
YAyFNGcSSPTAPLSPMSPPGYK	265-287	2435.7	2588.09	16	Y267
YAYFNGcSSPTAPLSPMSPPGyK	265-287	2435.7	2588.09	24	Y286
yAyFNGcSSPTAPLSPMSPPGYK	265-287	2435.7	2588.09	5	Y265/Y267
yAYFNGcSSPTAPLSPMSPPGyK	265-287	2435.7	2668.05	16	Y265/Y286
QASEQNWANySAEQNR	304-319	1895.9	1975.79	80	Y313

y- Phospho (+79.97 mass); c -Carbamidomethyl (+57.02 mass).

Table 13.1

Phosho-Tyr containing peptides of the Cx43CT, *in vitro* phosphorylated by ITK, identified from mass spectroscopy

13.4 Results

Tyr residues of Cx43CT phosphorylated by ITK as seen by an *in vitro* kinase assay.

The mass spectrometry data was validated by running a Western blot of the *in vitro* kinase reaction sample. The purified Cx43CT has six Tyr residues: Y247, Y265, Y267, Y286, Y301 and Y313. From the WT Cx43CT, six other constructs were prepared by mutation. Each construct had all the Tyr residues mutated to Phe (due to similarity in structure of Tyr and Phe and the latter lacking the hydroxyl group that is phosphorylated) except one Tyr. These constructs were individually subjected to an *in vitro* kinase reaction by incubating them with active ITK overnight. The overnight samples were resolved on polyacrylamide gel followed by Western blot analysis (Figure 13.1). Apart from Y301, all other Tyr show phosphorylation by ITK.

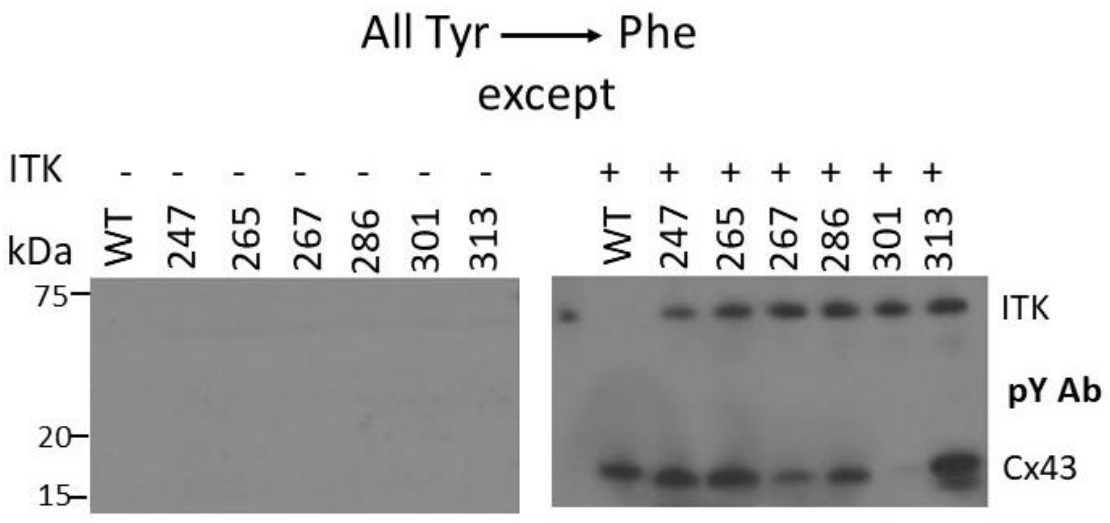


Figure 13.1. Identifying Cx43 Tyr residues phosphorylated by ITK using *in vitro* kinase assay: Purified Cx43CT was incubated with active ITK for the *in vitro* kinase reaction and then subjected to Western blot analysis using general phospho-tyrosine antibody. The lanes on the right of the WT indicate mutated constructs where all Tyr of the Cx43CT were mutated to Phe except the one mentioned on top of the lane. The control blot on the left had no active ITK added. Bands of phospho-Cx43 and phospho-ITK can be seen on the right blot.

Validation of Cx43 pY286 antibody

Among the Tyr residues identified by Western blot to be phosphorylated by ITK, Y286 is a novel site that has not been characterized previously. To validate the pY286 antibody, LA25 cells were used. These cells have been previously used to validate phospho-specific antibodies for Cx43 tyrosines [222]. LA25 cells have a temperature sensitive Src (inactive at 42˚C and active at 37˚C) and both versions were used for validation. Prior to Western blot and immunofluorescence analysis, antibody samples were incubated without and with non-phospho (Np) or phospho (p) peptide. The phospho peptide binds to the antibody and prevents it from binding to pY286 of Cx43. Data showed phosphorylation of Y286 with and without non-phospho peptide. However, no phosphorylation signal was noticed after blocking with phospho-peptide. Immunofluorescence data also showed similar results, indicating specificity of the antibody (Figure 13.2 A and 13.2 B).

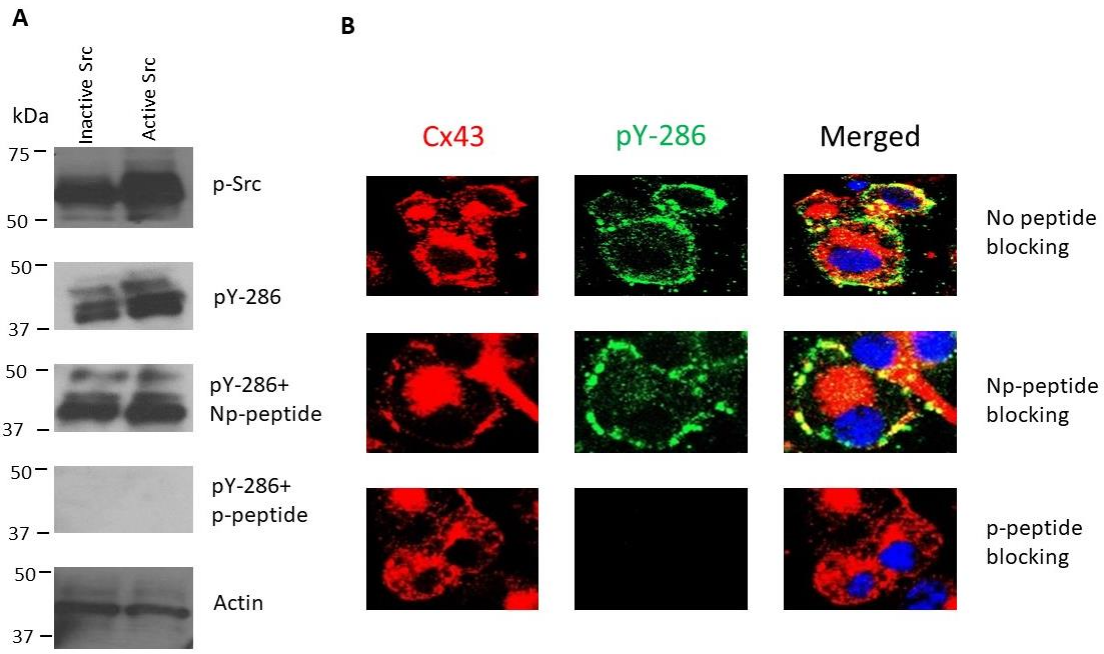
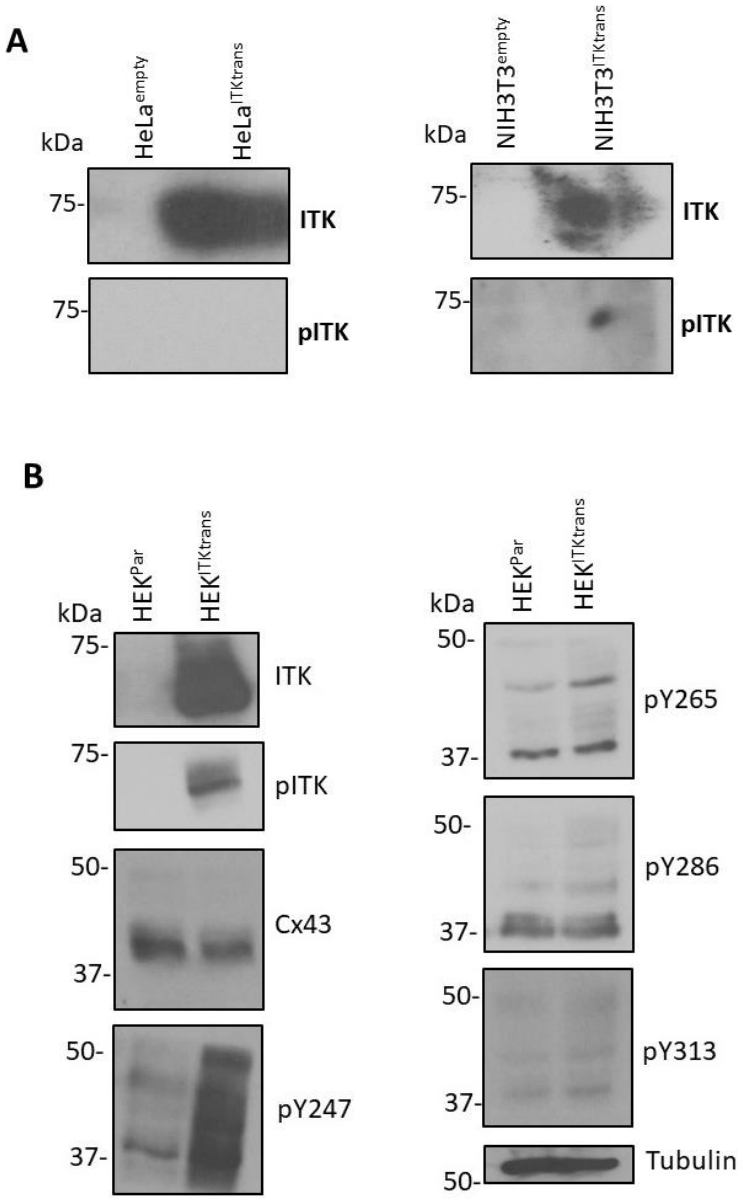


Figure 13.2. Validating the Cx43 pY286 antibody: LA25 cells were used to validate Cx43 pY286 antibody. A: On the left panel (Western blot), the antibody was incubated with without Non phospho-peptide (Np-peptide) or phospho peptide (p-peptide) and compared to the non-incubated control. B: Immunofluorescence data shows the same result of incubation of antibody with or without Np or p-peptide. Red: Cx43, Green: pY286, Blue: DAPI, Yellow: colocalization.

Phosphorylation of Cx43 Y247 increases in the presence of ITK

HeLa cells do not express Cx43 endogenously. This was a reason to use HeLa cells in our study as Cx43 can be manipulated by transfecting these cells with different (WT and mutated) forms of the connexin. In this study, HeLa cells stably expressing Cx43, were transiently transfected with ITK, incubated overnight after transfection, followed by lysis for Western blot analysis (Figure 13.3 A). ITK is activated by Tyr phosphorylation of its kinase domain. When the transfected HeLa samples were blotted with phospho-ITK (pITK) antibody, no signal was noticed. This was due to the lack of kinases that activate ITK including Lck. Thus, the focus was shifted to using cell lines that are known to have the kinases that activate ITK. Two such cell lines were chosen (that lack endogenous ITK) – NIH3T3 and HEK293T (HEK^{Par}). Both these cell lines were transiently transfected with ITK and analyzed by Western blot. NIH3T3 did not show activation of ITK (Figure 13.3 A), while HEK293T did (Figure 13.3 B). Thus, HEK cells were used for further experimentation. Western blot data showed increase in phosphorylation of Y247. Consequently, a cell line was generated for HEK^{Par} cells that stably expressed ITK (HEK^{ITK}). In HEK^{ITK}, the increase in the phosphorylation of Y247 was statistically significant. When HEK^{ITK} samples were blotted with Cx43 antibody, there was decrease in the P2 isoform along with increased P0 levels (Figure 13.3 C). Thus, overexpression of ITK affects the migration pattern of Cx43 and increases phosphorylation of Y247.


A
kDa
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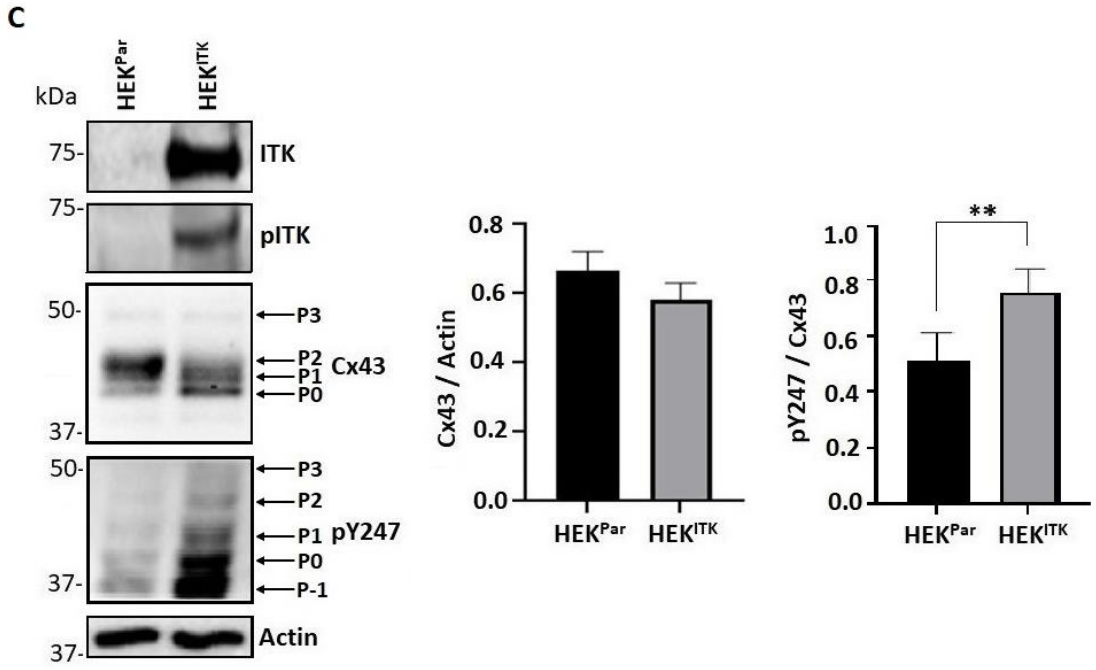

Figure 13.3. Effect of ITK on phosphorylation of Cx43 Tyr residues**:** Panel A: left picture shows ITK and pITK levels in HeLa cells transiently transfected with empty vector ($HeLa^{empty}$) and ITK plasmid ($HeLa^{ITKtrans}$); right picture shows ITK and pITK levels in NIH3T3 cells transiently transfected with empty vector ($NIH3T3^{empty}$) and ITK ($NIH3T3^{ITKtrans}$). Panel B indicates the Tyr phosphorylation of Cx43 by ITK in HEK^{Par} and HEK^{Par} cells transiently transfected with ITK ($HEK^{ITKtrans}$) using Western blot analysis. Panel C shows the same data in HEK^{Par} and HEK^{ITK} stable cells. Data was quantified with ImageJ and GraphPad prism with the help of Student's *t*-test (n=3, $^{**}p<0.01$).

ITK decreases Cx43 plaque size

When active ITK phosphorylates its substrates, it is present at the plasma membrane. To see its colocalization with Cx43, immunofluorescence was done. HEK^{Par} and HEK^{ITK} samples were stained with both Cx43 and ITK or phospho-ITK antibodies. Localization of Cx43 in HEK^{Par} cells was at the plasma membrane with little to none found intracellularly (Figure 13.4 left panel). In the HEK^{ITK} samples stained with Cx43 and ITK, it was hard to determine colocalization as ITK signal was present throughout the cell cytoplasm. In the stable cells stained with Cx43 and phospho-ITK, few regions were noticed to indicate colocalization. One noticeable difference between the parental and stable cell lines was the size of the Cx43 plaques at the membrane. The size of the plaque was significantly decreased in the presence of the kinase (Figure 13.4 right panel).

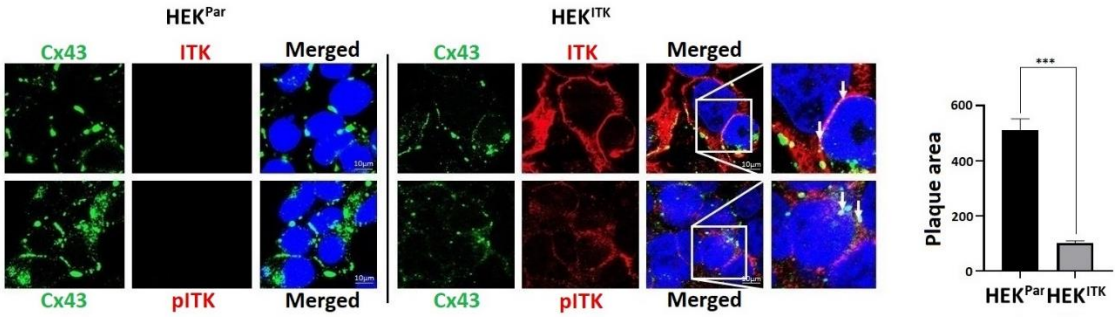

Figure 13.4. Studying effect of ITK on localization of Cx43 in HEK293T cells**:** Immunofluorescence was used to visualize Cx43 in HEK^{Par} and HEK^{ITK} cells. Green: Cx43, Red: ITK or phospho-ITK, Blue: DAPI. White arrows point to colocalization pockets of Cx43 with either ITK or pITK. Cx43 plaque area was quantified with ImageJ and GraphPad prism. (n=10, ***p<0.001).

ITK increases Cx43 levels that do not form part of the gap junction plaque

Gap junctional plaques are resistant to mild-detergent solubilization. Following Triton-X 100 extraction, ultracentrifugation separates the gap-junctional Cx43 (insoluble fraction) from the non-gap junctional Cx43 (soluble fraction). The assay result showed that soluble fraction was increased in the HEK^{ITK} samples compared to that in the control (Figure 13.5).

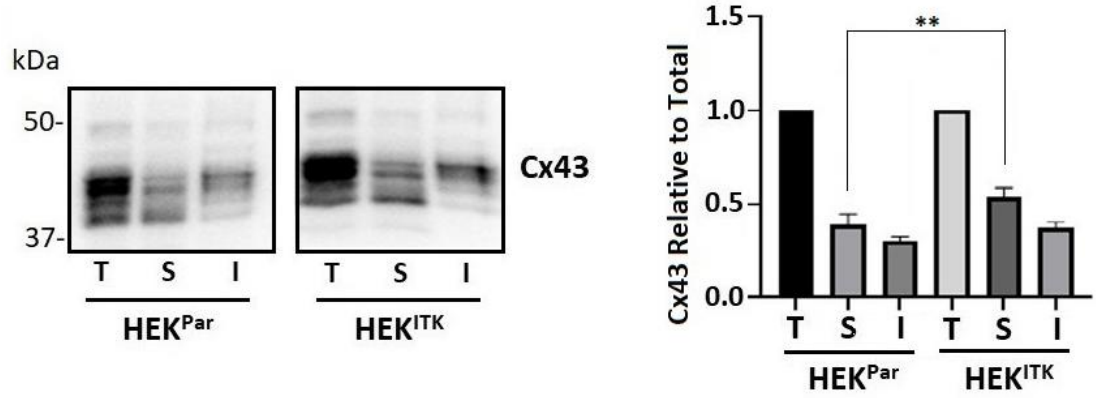

Figure 13.5. Effect of ITK on stability of Cx43 at the gap junction plaque: The mild detergent Triton X-100 was used to extract Cx43. Gap junctional fraction of Cx43 is referred to as insoluble (I), non-gap junctional fraction is soluble (S), total sample (T) includes insoluble (I) + soluble (S) fractions. Statistical analysis generated by ImageJ and GraphPad prism (n=3, **p<0.01).

ITK decreases GJIC in HEK cells

Lucifer Yellow (444 Da) is an anionic dye that can pass through Cx43 gap junction channels. With increase in GJIC, there is increase in the area of this dye spread through the channels. Scrape loading dye transfer assay was used to assess GJIC in HEK cells. The data shows a marked decrease in the dye transfer area in HEK^{ITK} samples (Figure 13.6). Less dye transfer indicates less GJIC. Unlike Lucifer Yellow, Texas Red Dextran has a higher molecular weight (3 kDa) which renders it gap junction impermeable and staining with this is used as a control to assess the data more effectively.

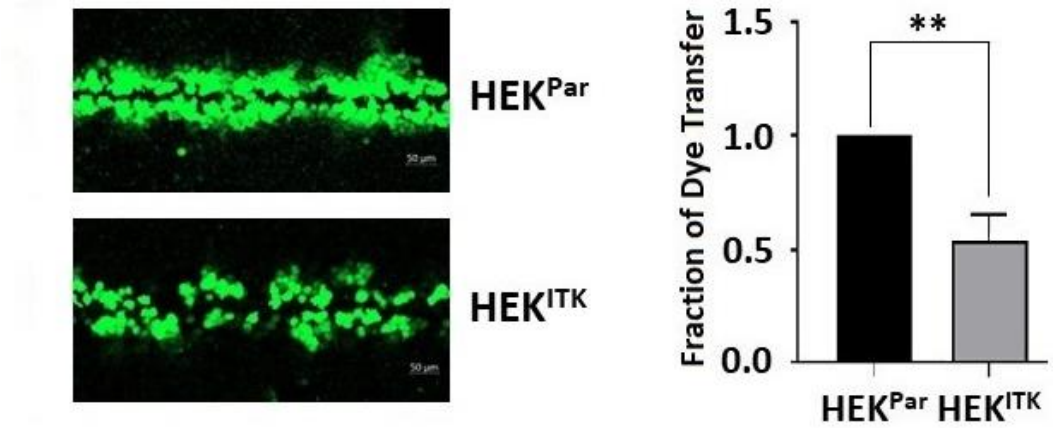

Figure 13.6. Effect of ITK on GJIC in HEK cells: Scrape loading dye transfer assay was used to study gap junction mediated intercellular communication (GJIC) in HEK^{Par} and HEK^{ITK} cells. Green: Lucifer Yellow. Area of dye transferred was measured by ImageJ and data was quantified by GraphPad prism (n=3, ****p<0.0001).

Activation of ITK increases Tyr phosphorylation of Cx43 in T lymphocytes

After using HEK cells to study the role of ITK in phosphorylation of Cx43, Jurkat cells (T lymphocytes) were used to determine if the results were consistent in a cell line that endogenously expresses ITK. Endogenous ITK was activated in Jurkat cells using α-CD3/α-CD28 antibodies. Activation of ITK was significantly increased. It corresponded with an increase in the pY247 levels along with that of the P3 and P4 bands compared to the non-activated sample (Figure 13.7). The migration pattern of Cx43 was unchanged compared to samples with non-activated ITK. The increase in the pY247 is consistent with that in HEK cells.

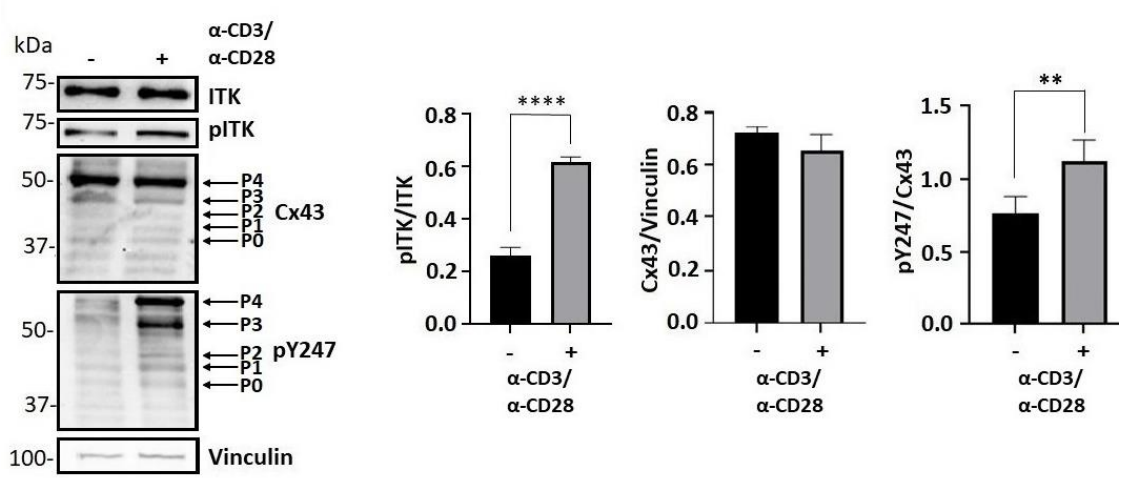

Figure 13.7. Effect of ITK on Cx43 phosphorylation in T lymphocytes: Jurkat cells were used for Western blot analysis of Cx43 phosphorylation as a result of ITK activation through the T-cell receptors. Arrows point to the different migrating isoforms of Cx43. Band signals were measured by ImageJ, followed by quantification with GraphPad prism (n=3, ****p<0.0001).

In the immunofluorescence data, there is a lower phospho-ITK level in the non-activated cells. ITK activation also shows the presence of few colocalization pockets of Cx43 and active ITK (Figure 13.8).

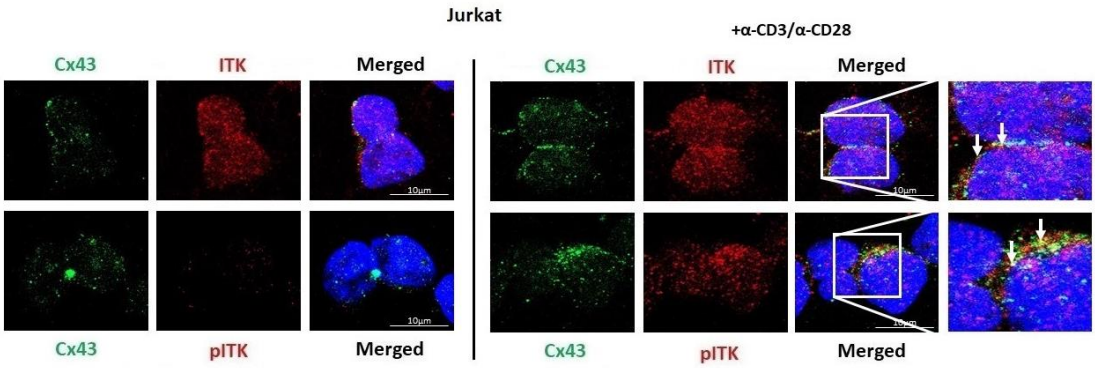

Figure 13.8. Effect of active ITK on cellular localization of Cx43 in T lymphocytes: Immunofluorescence data was used to visualize localization of endogenous Cx43 in Jurkat cells with and without activation of ITK. Green: Cx43, Red: ITK or pITK, Blue: DAPI. White arrows indicate pockets of colocalization of Cx43 with ITK or pITK.

Activation of ITK decreases GJIC

Since Jurkat cells are non-adherent cells, parachute assay technique was used instead of scrape loading dye transfer assay to assess GJIC. For each replicate of the experiments, there was one donor plate (cells loaded with both DIL and calcein-AM) and two recipient plates (unloaded cells for non-activated and activated). Few cells from the donor plate were dropped onto both recipient plates and incubated for the indicated time points. During this time, the calcein (green) from the donor cells is transferred to the recipient cells only through gap junctions. A higher number of green recipient cells around one donor cell indicates increased dye transfer, which in turn indicates more GJIC.

Activation of ITK causes significant decrease in the number of recipient cells surrounding one donor cell, thus indicting decrease in GJIC (Figure 13.9).

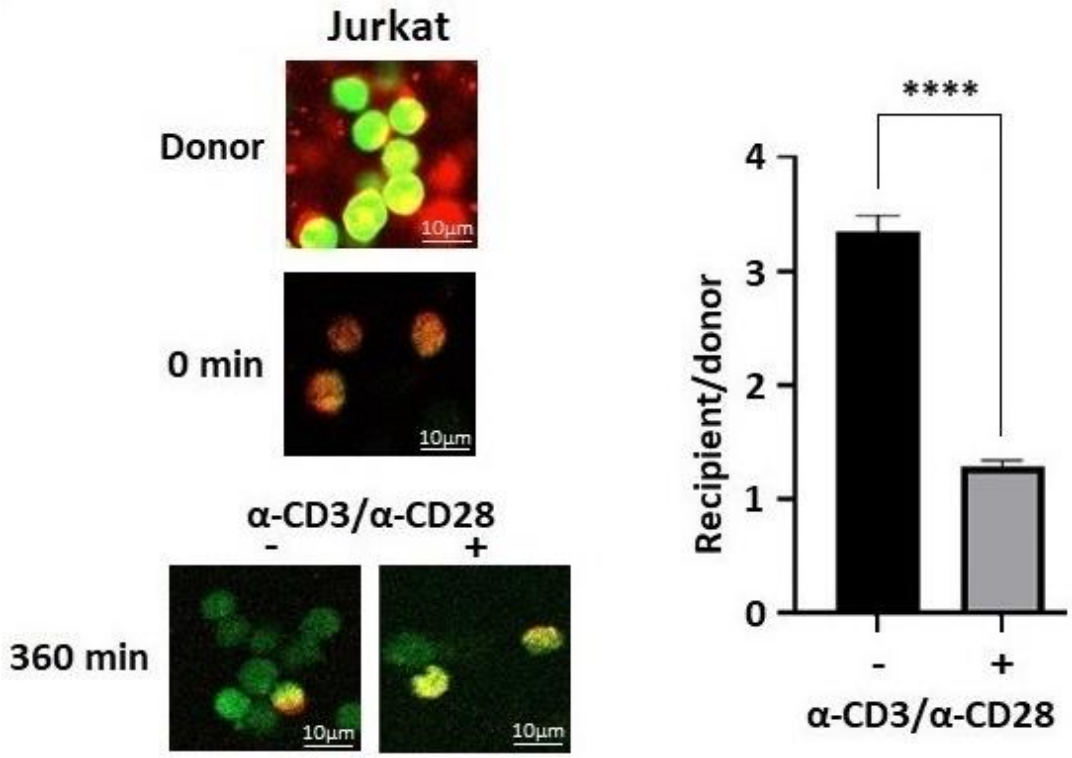

Figure 13.9. Effect of active ITK on GJIC in T lymphocytes**:** Parachute dye coupling assay was used to determine GJIC in Jurkat cells with and without ITK activation. Red/Yellow: Donor cells, Green: Recipient cells that have taken up the dye calcein from the donor cells. Statistical analysis was done using GraphPad prism (n=3, ****p<0.0001).

13.5 Discussion

Cx43 is ubiquitously expressed in immune cells and its expression is the highest compared to other connexin isoforms in T lymphocytes. Cx43 is involved in proliferation of splenic T cells and cytokine production [502]. Cx43 gap junctions accumulate at the T cell immunological synapse promoting the killing of tumor cells [503]. Cx43 is also important for the maintenance of T cell homeostasis [504].

Gap junctions are important for cell-cell communication involving T cells. Gap junction channels play key roles in activation of T cells after antigen presentation [499]. T cell maturation in the thymus requires gap junction [301]. Cx43 gap junctions are involved in the proliferation and differentiation of matured T cells [499]. Accumulation of gap junctions at immunological synapse is necessary for T cell activation [505]. Differentiation of T cells has been shown to be suppressed by using gap junction inhibitors [506]. Normal migrating T cells have low levels of gap junctions and their increase is noticed in peripheral blood T cells during hypertension [507]. In tumor conditions, upon reaching target cells, gap junctional communication between cytotoxic T cells and tumor cells is necessary to kill target tumor cells [508]. Thus, regulation of gap junction activity in T lymphocytes is necessary to help mediate a T cell mediated adaptive immune response.

Migration pattern of Cx43 was altered in presence of ITK in HEK cells. Following overexpression of ITK, a higher intensity of P0 and P1 Cx43 bands was noticed along with a lower intensity of P2 band compared to the control sample. This change in migratory pattern is consistent with our data from overexpression of BTK in HEK cells. In presence of ITK, increase in phosphorylation of Y247 was significant. The phosphorylation of Y265, Y286, Y313 were not significant although mass spectrometry had identified these as

residues to be phosphorylated by ITK. This can be attributed to a network of various protein molecules involved inside a cellular system as opposed to *in vitro* systems. In case of phosphor-Y247, there is an overall increase in the intensity of all migratory isoforms, with the increase in P-1 and P0 being the most prominent. As mentioned in literature [221, 239], the faster migratory isoforms of Cx43, in this case the P-1, P0 and P1 bands correspond to Cx43 that do not form part of a gap junction. On the other hand, the slower migrating P2 isoform corresponds to Cx43 that belong to gap junction plaque. Studying the migratory patterns, our Western blot data suggests an overall increase in non-gap junctional Cx43. Localization study of Cx43 using immunofluorescence technique showed no change in the position of Cx43. In both presence and absence of ITK, Cx43 is present mostly at the plasma membrane. However, the size of Cx43 plaque at the plasma membrane shows a significant decrease in presence of ITK. This suggests that ITK is causing dissociation of Cx43 from the gap junction plaque, without affecting its internalization or disruption from the plasma membrane itself. Our data from BTK overexpression in HEK cells also showed similar results. TX-100 solubility assay shows a significant increase in the levels of Cx43 present in the non-gap junctional region of the plasma membrane, which is also consistent with the Western blot, immunofluorescence and BTK overexpression data. However, the soluble to insoluble ratio is not significantly increased as a decrease in the insoluble (gap junctional Cx43) fraction is not noticed. It is possible that overexpression of ITK may increase the activation of another factor in the cell that is causing an increase in the overall association of Cx43 to the plasma membrane. This may render the decrease in gap junctional Cx43 to be undetectable by TX-100 assay (which is less sensitive than Western blot). The migratory pattern of different Cx43 isoforms does not change in Jurkat cells after

ITK activation unlike in HEK cells. This dissimilarity may occur due to use of different cell types and also due to the fact that ITK in Jurkat cells was endogenous while it was overexpressed in HEK cells. However, the increase in the overall intensity of the Cx43 phospho-Y247 bands is consistent across both cell types. Localization of Cx43 does not change with or without ITK, as in both cases it is present in the plasma membrane. There is evidence suggesting the role of ZO-1 in regulating plaque size and gap junction assembly [7, 268]. The absence of ZO-1 in Jurkat cells may be a reason for no change in plaque size. We noticed a marked decrease in GJIC as a result of ITK activation in both HEK cells and Jurkat cells. Thus, ITK affects the stability of Cx43 at the gap junction plaque and causes decrease in GJIC. Since our immunofluorescence data shows no increase in internalization of Cx43, it is suggested that the decrease in GJIC may be caused due to closure of channels. Cx43 phosphorylation regulates conductivity of a channel and can affect its open and closed state [290, 489-491]. This study indicates a role for ITK in affecting the gating state of Cx43 gap junction channels.

CHAPTER FOUR

Conclusion and future directions

14.1 Conclusion

Phosphorylation of Ser, Thr or Tyr residues in the Cx43CT can affect the functioning of gap junction channels in various ways. Some of these include changing the permeability of the channels, altering the gating properties or causing internalization and turnover of connexins [105, 208, 222, 228, 250, 290, 292, 489]. Phosphorylation of Y247, Y265 and Y313 by Src causes disassociation of Cx43 from cytoskeletal elements and decreases GJIC through internalization [222]. By screening the purified Cx43CT against kinases, two novel kinases: BTK and ITK were found to phosphorylate the Cx43CT. Both these kinases are expressed in the immune system. Although expressed by many immune cell types, the role of BTK has been majorly studied in the context of B lymphocytes [406]. ITK is known to be expressed in T lymphocytes [435]. BTK and ITK also possess structural and functional similarities to Src [397, 441, 509-512]. Since B and T cells have little to no expression of Src, we hypothesized that BTK and ITK may play a compensatory role in B and T cells respectively by phosphorylating Cx43CT and decreasing GJIC.

Mass spectrometry analysis showed BTK and ITK to phosphorylate Tyr residues in Cx43CT. To validate this data, we did an *in vitro* kinase reaction of Cx43CT with either BTK or ITK and analyzed the samples using Western blot. This data indicated the differential intensity of phosphorylation of the Tyr residues in Cx43CT. We then shifted the phosphorylation assays from an *in vitro* system to a cellular system. BTK and ITK were overexpressed in HEK293T (HEK) cells. This caused changes in the migration pattern of Cx43 on the polyacrylamide gel. The slower migrating P2 bands decreased in intensity while the faster migrating P0 and P1 bands increased. This indicates an increase in the Cx43 that do not form part of gap junction plaque. Both the kinases significantly increased

the phosphorylation of Y247. We repeated this study in Daudi (B cells) and Jurkat (T cells) after activating BTK and ITK respectively. Although the Cx43 migration pattern did not change as a result of kinase activation, phospho-Y247 levels were significantly increased as seen in HEK cells in the presence of the kinases. Immunofluorescence data showed BTK and ITK to affect the size of the gap junction plaque at the plasma membrane. The plaque size significantly decreased in presence of the kinases in HEK cells. This also indicates that BTK and ITK affect the stability of Cx43 at the gap junction plaque. In both HEK and immune cells, Cx43 is present mostly at the plasma membrane with or without kinase activity. Thus, BTK and ITK do not seem to affect the cellular localization of Cx43. TX-100 solubility assay was used which helps us to compare the levels of Cx43 at the gap junction plaque with that of Cx43 that are not part of the plaque. In HEK cells, the results of this assay show a significant increase in the non-gap junctional Cx43 levels following BTK and ITK kinase activity. With BTK activation, there is also a significant decrease in the Cx43 levels present at the gap junctional plaque. This is also consistent with the Western blot and immunofluorescence data which indicate role of BTK and ITK in affecting the stability of Cx43 at the gap junctional plaque. Finally, scrape loading dye transfer assay and parachute assay were used in HEK cells and immune cells respectively to study the effect of BTK and ITK on GJIC. Both the kinases significantly decrease GJIC in HEK as well as immune cells. Analyzing the results of this study, we can conclude that BTK and ITK affect Cx43 stability at the plaque and decrease GJIC by probably affecting the gating properties and causing closure of gap junction channels.

14.2 Future directions

14.2.1 A major event that occurs as part of B and T cell mediated adaptive immune response is the migration of these lymphocytes from one tissue type to another [513]. As gap junctions facilitate cell-cell communication, migration studies have been conducted to find a link between Cx43 and motility of cells. Glioma cells have shown increased motility due to reduced Cx43 expression [480]. In another study, Cx43 has been shown to play a role in increasing adhesiveness of HeLa cells and decreasing their motility [481]. It will be interesting to see if peripheral blood lymphocytes show reduced Cx43 expression and GJIC. Do BTK and ITK activities increase in migrating lymphocytes, and do they affect GJIC through phosphorylation of Cx43?

14.2.2 Evidences show GJIC to exist between lymphocytes and target tissue such as cardiomyocytes in heart diseases [514]. Upon reaching the target organs, B and T cells need to communicate with other cell types to elucidate adaptive immune response. From our data and the literature, we hypothesize that BTK and ITK activities may be reduced in B and T cells respectively after they have reached their target tissues. This may be necessary in order to reduce Cx43 phosphorylation and increase GJIC to facilitate communication of B and T cells with other cells. Co-culturing tissues such as cardiomyocytes with lymphocytes can help us to understand the effect of BTK and ITK on Cx43 phosphorylation and GJIC in target organs.

14.2.3 There are a few mechanisms involved in regulating GJIC, including connexin phosphorylation mediated internalization, change in channel permeability and closure of channels. In our study, we noticed no change in cellular localization of Cx43 after phosphorylation by BTK or ITK. Thus, we hypothesize that the decrease in GJIC may be

due to channel closure. Knowing the mechanism behind the decrease in GJIC is necessary to better comprehend the effect of BTK or ITK on Cx43 phosphorylation and regulation.

14.2.4 Hyperphosphorylation of Cx43 bands in the immune cells and its change in migration pattern indicate the presence of Ser phosphorylation besides Tyr phosphorylation. If PKC is being activated as a result of BTK or ITK activation, then phosphorylation of Cx43 by PKC will lead to increased selective permeability of the channel. Studying which molecules are being prevented from passing through, we can understand the further downstream effect of BTK or ITK activation on Cx43 channel functioning. For example, if increased selective permeability leads to prevention of certain miRNAs through the channel then it can alter the transcriptional scenario of the cell as miRNAs are known to be transcriptional regulators.

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