THE CONTRIBUTION OF DIFFERENT DOMAINS OF CONNEXIN 43 TO CYTOSKELETAL REARRANGEMENTS IN B-LYMPHOCYTES

by

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ABSTRACT

B-cells change shape in response to crosslinking of the B-cell antigen receptor (BCR). BCR signaling induces cytoskeletal rearrangements that result in cell spreading to improve antigen accumulation and B-cell activation. It has previously been shown that the gap junction protein connexin43 (Cx43) is both necessary and sufficient for BCR-mediated B-cell spreading, as well as other B-cell responses that depend upon cytoskeletal rearrangement. Since it was found that the C-terminal domain (CT) of Cx43 was required for these effects, we hypothesized that the molecular mechanism by which the CT influences BCR-mediated spreading may be due to regulation of channel permeability or alternately, by acting as an adaptor for cytoskeletal organization.

To address the role of Cx43 in forming channels, we blocked channel function both pharmacologically with channel-blocking drugs (carbenoxolone, probenecid, and lanthanum) and genetically by expressing Cx43 mutant Cx43T154A in a Cx43-null plasmacytoma cell line that had been previously transduced to express the BCR (J558μm3) for use in spreading assays. Treatment with channel blocking drugs did not prevent BCR-mediated B-cell spreading, and hemicchannel (HC) activity was not detected in B-cells as measured by a dye-uptake assay. Thus we conclude that Cx43 influences B-cell spreading by a channel-independent mechanism and that the Cx43 CT may act as a scaffold for protein interactions involved in cytoskeletal rearrangement downstream of BCR signaling. In support of this idea, the channel-blocking point mutation Cx43T154A caused B-cell spreading defects even in the absence of functional HCs. Further characterization of this mutation suggests that it impedes normal BCR-mediated cell spreading due to a distorted conformation of the Cx43 CT domain.
To further investigate the importance of the Cx43 CT domain, the putative Src-binding residue tyrosine 265 was mutated. J558µm3 cells expressing Cx43 with the single point mutation Cx43Y265F or Cx43Y265D were unable to spread, highlighting the importance of a single residue of Cx43 for BCR-mediated B-cell spreading. These findings highlight the CT domain as important for Cx43’s influence on B-cell spreading, and pave the way for further experiments on the CT tail with the goal of better understanding the molecular mechanism underlying the role of Cx43 in B cells and in the immune response in general.
PREFACE

I Collaboration

Data from Figure 3.2 was collected in collaboration with Dr. Jose Luis Vega Pizarro, visiting Postdoctoral Fellow, Universidad de Catolica de Chile, Santiago Chile, with Dr. Christian Naus lab, Department of Cellular and Physiological Sciences, UBC. Data from Figure 3.9 was collected by May Dang-Lawson.

II UBC research ethics board certificates

UBC Animal license and breeding program #A10-0384
Matsuuchi Lab animal license #A11-0317.
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<td>α</td>
<td>Anti-</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein-2/3</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Ca²⁺</td>
<td>Calcium</td>
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<td>CBX</td>
<td>Carbenoxolone</td>
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<td>CIP</td>
<td>Calf intestinal phosphatase</td>
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<td>CK-1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CL</td>
<td>Cytoplasmic loop</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxyl-terminal</td>
</tr>
<tr>
<td>cSMAC</td>
<td>Central supermolecular activation complex</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>DCFS</td>
<td>Divalent cation free solution</td>
</tr>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
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<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Extracellular</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td><em>Escherichia coli</em></td>
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</tr>
<tr>
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<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>EtBr</td>
<td>Ethidium bromide</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
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<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Guanine exchange factor</td>
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<td>GJ</td>
<td>Gap junction</td>
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<td>Ix</td>
<td>Innexin</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<td>La&lt;sup&gt;3+&lt;/sup&gt;</td>
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<td>LB</td>
<td>Lysogeny broth</td>
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<td>LFA-1</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
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<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td>Magnesium sulphate</td>
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<td>Major histocompatibility complex</td>
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mlg Membrane Ig
mRNA Messenger RNA
MW Molecular weight
Na$_2$HPO$_4$ Sodium phosphate
Na$_3$VO$_4$ Sodium pervanadate
NaCl Sodium chloride
NMR Nuclear Magnetic Resonance
NT Amino-terminus
ODDD Oculodentodigital dysplasia
Pbn Probenecid
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PE Phycoerythrin
PI3K Phosphoinositol-3 kinase
PKA Protein kinase A
PKC Protein kinase C
PLC$_\gamma$ Phospholipase C$_\gamma$
PM Plasma membrane
PMSF Phenylmethylsulfonyl fluoride
pSMAC Peripheral supermolecular activation complex
pY Phospho-tyrosine
Panx Pannexin
rpm Revolutions per minute
RPMI Roswell Park Memorial Institute
SCAM Substituted cysteine accessibility method
SDS-PAGE Sodium dodecylsulfate polyacrylamide gel electrophoresis
sec Seconds
SH2 Src homology 2
siRNA Small interfering RNA
T Threonine
TBS Tris-buffered saline
TBST TBS + 0.1% Tween-20
TCR T-cell antigen receptor
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<td>Untranslated region</td>
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<td>Walter and Eliza Hall Institute</td>
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<td>WT</td>
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<td>Tyrosine</td>
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CHAPTER 1
INTRODUCTION

1.1 The immune system

The defining characteristic of immunity is the discrimination between self and non-self. The immune system is evolutionarily conserved among metazoans since multicellular organisms need to protect themselves from invasion by disease-causing viruses, bacteria, fungi and parasites, known collectively as pathogens. In addition, multicellularity requires a great degree of coordination, and immunity allows for the recognition of aberrant self when cells become dysregulated such as in cancer. In mammals, the immune system has evolved to have two branches: the quick responding innate immune response and the slower and more specific adaptive immune response.

The innate immune system is evolutionary ancient and faster acting than the adaptive immune system and acts as the first line of defense against infection. Innate immunity includes mechanical, proteinaceous, and cellular mechanisms to prevent infection from occurring in the first place, and to trap and clear pathogens that successfully invade the host. Mechanical barriers include the epithelium and the resident flora that line it, the continuous flushing of surfaces by fluids like tears, urine, and mucous, and actions that clear trapped pathogens like coughing and sneezing. Proteinaceous factors are non-cellular macromolecules found in the blood plasma, interstitial fluid, and lymph, including proteins of
the complement system, cytokines, and anti-microbial peptides secreted by cells of the innate immune response. Innate immune cells (neutrophils, eosinophils, basophils, and macrophages) recognize common pathogenic patterns such as bacterial cell compounds like lipopolysaccharide (LPS), peptidoglycan, flagellin, double stranded RNA, and unmethylated CpG sites of DNA, as well as nuclear and cytosolic components of host cells exposed by necrotic damage through evolutionarily conserved pattern recognition receptors such as toll-like receptors (TLRs), and C-type lectin receptors (CTLRs) and kill pathogens by phagocytosis or by the release of anti-microbial factors from cytotoxic granules (Akira et al., 2006).

Unlike the innate immune response, which is limited to recognition of commonly occurring pathogenic patterns by non-specific receptors, the adaptive immune response has the ability to recognize a nearly unlimited variety of pathogens by the generation of specialized receptors through genetic recombination. The main players in the adaptive response are a specialized group of leukocytes, or white blood cells, called lymphocytes. T- and B-lymphocytes each bear a receptor on their cell surface with a unique receptor specificity, which upon recognition of its cognate ligand or “antigen” (Ag) leads to activation and clonal expansion of that single T- or B-cell. Clonal expansion leads to proliferation and to somatic hypermutation of the B-cell antigen receptor (BCR) to fine-tune its specificity through affinity maturation. Mature plasma B-cells secrete Abs that neutralize or opsonize pathogens for destruction and mature T-cells kill targeted cells and regulate the immune response by secreting cytokines that regulate the activities of cells of both the innate and adaptive immune response. Clonal expansion also leads to the generation of memory cells, which can
be retained for years and contribute to a quicker response in the case of re-infection by the same pathogen (Murphy et al., 2011).

1.2 B-lymphocytes

1.2.1 B-lymphocytes in the immune response

B-lymphocytes develop in the bone marrow from hematopoietic stem cells through interaction with stromal and reticular cells. B-cell development is largely categorized based upon maturation state of the BCR. Naïve or “immature” B-cells released from the bone marrow express membrane bound BCR of the Immunoglobulin M (IgM) isotype on their cell surface (Yuan and Witte, 1988). These cells migrate through the blood, lymph, and secondary lymphoid organs where they perform the role of immune surveillance by sampling environments searching for Ags (Murphy et al., 2011).

Recognition of Ag by BCR leads to B-cell activation, proliferation and differentiation through altered gene expression. Activated B-cells undergo somatic hypermutation which leads to affinity maturation of the BCR, during which class-switching may also take place. During class-switching, the BCR heavy chain, which is the isotype-determining component, may be changed from the immature IgM to another type (Pascual et al., 1994). There are five different BCR heavy chains: IgM, IgD, IgG, IgA, and IgE, which differ primarily in their properties in secreted form, however this discussion will focus mainly on IgM (Chapter 1.2.2). Clonal expansion leads to two functionally distinct populations of mature B-cells: plasma cells and
memory cells. Plasma cells secrete antibodies (Abs) that harbor the same Ag specificity as the BCR and are derived from a splice variant of the same gene that lacks the transmembrane domain that anchors the BCR on the cell surface. Secreted Abs coat pathogens, which opsonizes them for destruction by the complement system or by effector cells that can bind Abs through Fc receptors. Memory B-cells, expressing BCRs on their cell surfaces, are retained in the bone-marrow and can be quickly re-activated by their cognate Ag binding to the BCRs, leading to differentiation into Ab-secreting plasma cells once more.

B-cell activation also leads to Ag-internalization. The affinity and amount of Ag-binding determines the likelihood of B-cell activation (Batista and Neuberger, 2000), but internalized antigen is also processed for cross-presentation on major histocompatibility complex II (MHC II) molecules for the stimulation of helper T cells (Lanzavecchia, 1985). Helper T cells prime the immune response by secreting cytokines that act on many immune cells and assist in B-cell class-switching.

1.2.2 Structure of the BCR

While secreted Abs have been studied for over a century, the surface bound form (the BCR) was not identified on B-lymphocytes until the 1970’s. That the BCR was a cell surface-bound immunoglobulin (Ig) was determined by experiments showing the transformative potential of anti-Ig antisera to stimulate rabbit lymphocytes (Sell and Gell, 1965) and by immunofluorescent labeling of membrane Ig (mIg) (MC RAFF and Taylor, 1970; Pernis et al., 1971).
Structurally, the BCR consists of an Ag-binding subunit and a signaling subunit (for review see (Reth, 1995)). The Ag-binding subunit is composed of two Ig chains linked through disulfide bonds. The Ig chains are a heavy chain and a light chain named for their relative molecular weights. There are two types of light chain, lambda (λ) and kappa (κ), which have a molecular weight of approximately 25-28 kDa and whose variable region genes are recombined to create the unique specificity of each BCR. There are five heavy chain isotypes, IgM, IgD, IgA, IgG and IgE. This discussion will focus only on the IgM isotype which is 67-78 kDa (Vitetta et al., 1971). The variable region of the genes encoding the heavy chains are also recombined to generate unique binding sites that generate the BCR specificity when combined with the light chains that have gone through the same process.

Early studies in B-cell lines showed that mlgM was not the entire BCR but that the Ag-binding subunit formed a complex with a signaling subunit that was required for trafficking of BCR to the cell surface (Matsuuchi et al., 1992). The signaling subunit is a disulfide-linked heterodimer composed of two Ig protein superfamily members: Igα and Igβ. Igα is a 34 kDa protein encoded by the gene mb-1 (Hombach et al., 1988), and Igβ is a 39 kDa protein encoded by the gene B29 (Hermanson et al., 1988). Igα and Igβ have cytoplasmic carboxy-terminal tails which contain immunoreceptor tyrosine-based activation motifs (ITAMs) which are phosphorylated upon BCR stimulation and initiate the BCR signaling cascade. The Ag-binding and signaling subunits oligomerize in the ER and are trafficked to the cell surface as a protein complex (Figure 1.1).
Figure 1.1 Structure of the B-cell antigen receptor (BCR) in the plasma membrane. BCR of the J558μm3 cell line used most commonly throughout this project, composed of the Ag-binding subunit: mlgM, composed of heavy chain Igμ (green) and light chain Igλ (orange) held together by disulfide bonds shown in black bars, and the signaling subunit composed of Igα (red) and Igβ (blue) which contain ITAMs (purple) and tyrosine residues (yellow circles).
1.2.3 BCR-mediated cell spreading

The BCR is divalent so crosslinking of BCR molecules into larger aggregates by Ag-binding activates the BCR and leads to the formation of BCR signaling clusters in the membrane (Depoil et al., 2007; Fleire et al., 2006). BCR crosslinking leads to the phosphorylation of ITAMs within the Igα and Igβ cytoplasmic domains, likely by nearby Src family kinases such as Lyn, Fyn, Blk, and Lck (Gold et al., 1990). Phosphorylated tyrosine residues in these ITAM domains recruit non-receptor tyrosine kinase Syk which can bind these residues through its SH2 domain (Takata et al., 1994). This leads to subsequent phosphorylation events that activate a cascade of kinases leading to three main signaling pathways mediated by the following enzymes: PLCγ, Ras/MAPK, and PI3K (for review of BCR signaling see (Defranco et al., 2006). These pathways lead to changes in gene expression resulting in either B-cell proliferation, or to cell death if the B-cell is at a specific stage of development.

In addition to activating transcription factors that cause changes to gene expression, BCR signaling results in more immediate and proximal modifications to the actin cytoskeleton. While early studies of BCR-signaling were performed biochemically, advances in microscopy techniques have allowed for the visualization of early events at the plasma membrane during BCR-Ag binding. F.D. Batista and others have shown that the B-cell undergoes a dynamic spreading and contraction response upon BCR stimulation which facilitates the formation of the immune synapse (Fleire et al., 2006). The immune synapse forms between the B-cell and APC and consists of a central cluster of Ag-BCR called the central supermolecular activation
**Figure 1.2 BCR-mediated B-cell spreading leads to formation of the immune synapse.**

BCR stimulation by recognition of its ligand/Ag results in B-cell spreading and then contraction which allows the B-cell to accumulate Ag (red dot), form BCR micro-clusters (green), and form an immune synapse which consists of a signaling center containing BCR and Ag called the central supramolecular activation cluster (cSMAC), surrounded by the peripheral supramolecular activation cluster (pSMAC) which is composed of adhesion molecules such as integrins (purple). The B-cell surface is depicted independently of antigen presenting cell surface or lipid bilayer upon which the B-cell spreads. The immune synapse facilitates prolonged BCR signaling and Ag-internalization.
complex (cSMAC), surrounded by a ring of integrins called the peripheral supermolecular activation complex (pSMAC) (Figure 1.2). The immune synapse facilitates prolonged BCR-signaling and Ag-internalization (Carrasco et al., 2004).

This B-cell spreading response depends upon a wave of actin depolymerization that is required for the reorganization of the cytoskeleton within lamellopodia for cell spreading. Breakdown of the cortical actin cytoskeleton was also recently demonstrated to be sufficient to increase BCR mobility and lead to BCR cluster-formation and signaling (Treanor et al., 2011).

Our knowledge of the signaling pathway leading from BCR activation to cytoskeletal rearrangement is incomplete, however the Rap1 GTPase has emerged as a key effector for regulation of cytoskeletal dynamics downstream of the BCR. Rap1 activity is important for B-cell spreading and formation of the immune synapse (Lin et al., 2008a; McLeod et al., 1998; McLeod et al., 2004). The GTPase activity of Rap1 allows it to act as a molecular switch and regulate the activity of a variety of actin-binding and regulating proteins. The GTP bound form of Rap1 is active but can be converted to Rap1-GDP by GTPase activating proteins (GAPs), which help catalyze the hydrolysis of GTP. Guanine exchange factors (GEFs) convert Rap1-GDP back into active Rap1-GTP (Figure 1.3). Overexpression of a constitutively active Rap-specific GAP (RapGAPII) in the A20 B-cell line prevented BCR-mediated B-cell spreading, demonstrating Rap1’s importance in this process (Lin et al., 2008b).
Figure 1.3 Activities of the Rap1 GTPase. The Rap1 GTPase acts as a molecular switch to control rearrangements of the cytoskeleton by activating various actin binding or actin regulating proteins (colored rectangles). Rap1 activity is controlled by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) that facilitate the addition of a phosphate group or hydrolysis of GTP respectively. Rap1 is activated by signaling cascades that result from BCR crosslinking, chemokine receptor ligand binding, and integrin engagement. Rap1 also contributes to inside-out signaling: causing integrins to adopt an activated conformation and resulting in breakdown of the cortical actin cytoskeleton to enhance BCR mobility and therefore BCR cluster formation independently of BCR signaling.
1.3 Connexins

1.3.1 Structure and formation of gap junctions and hemichannels

Cell-cell communication is important to all multicellular organisms for the coordination of cellular processes and propagation of signals through tissues. The “gap junction” (GJ) is a plaque of protein channels that connects adjacent cells whose membranes remain separated by a 2 nm “gap”. GJ plaques were first identified in electron micrographs of the axons of goldfish (Robertson, 1963) and were first referred to as “Gap Junctions” by Revel and Karnovsky (Revel and Karnovsky, 1967) to differentiate them from other junction types like adherens and tight junctions. In vertebrates, each GJ channel is made up of twelve protein subunits called “connexins” (Cxs), a name that was first used to describe the protein family by D. A. Goodenough in 1987.

GJ channels allow for the passive diffusion of small ions and metabolites of up to 1 kDa in size between coupled cells. This communication is important for many physiological processes ranging from myocardial contraction (Barr et al., 1965), vasomotor tone (Christ et al., 1996), and smooth muscle contraction (Campos et al., 1993; Daniel et al., 2001; Garfield et al., 1988), to cell cycle regulation (Kardami et al., 2007) and cellular differentiation (Trosko et al., 2000). GJs can also suppress defects resulting from somatic mutations and may act as tumor suppressors, although GJs have conflicting roles in tumorogenesis, acting as both tumor suppressors (Mesnil, 2002; Naus and Laird, 2010) but also increasing tumor invasion of some cancer cell types (Ezumi et al., 2008; Li et al., 2007; Lin et al., 2002).
Strategies for cell-cell communication have evolved independently in different multicellular organisms. Plant cells communicate through plasmodesmata, which differ structurally from GJs due to the presence of cell walls in plants. Fungal cells sometimes connect through “pores” though they frequently form fused cell structures since fungi form less organized tissues in general. Within the animal kingdom, GJ proteins evolved in at least two independent events by convergent evolution, giving rise to the innexin (Ix) family of GJ proteins in invertebrates and the connexin (Cx) family in vertebrates, which share structural but not sequence homology (Scemes et al., 2009). A more recently discovered pannexin (Panx) family of proteins found in vertebrates share sequence homology with the invertebrate Ixs, providing further support that the Ixs are the more ancestral GJ-forming protein family, and that they have been adapted to hemichannel (HC) function in vertebrates. Panxs function exclusively as HCs in vertebrates due to the glycosylation of the extracellular loops. These modifications may sterically hinder docking of HCs into GJs in vertebrates since no other GJ proteins are found to be glycosylated on the extracellular loops, and since close proximity is required for disulfide linkage of GJs, these types of modifications inhibit proper GJ formation (Sosinsky et al., 2011).

There are 20 different Cx proteins in mice and 21 in humans, named for their molecular weight as determined by mobility using SDS-gel electrophoresis since these proteins differ markedly in length, especially of their cytoplasmic carboxyl-terminal (CT) domains (for review see (Sosinsky and Nicholson, 2005). Predictions based on sequences of isolated Cx cDNAs, proteolysis, and mapping using antisera of different specificities, has led to models of
GJ structure that have since been confirmed by crystallography (Unger et al., 1999) and nuclear magnetic resonance (NMR) experiments (Sorgen et al., 2004). Cx family members all share a high degree of conservation in their four transmembrane domains (TM1-4). These TM domains are connected by two extracellular loops E1 and E2. Each extracellular loop contains three conserved cysteine residues that form disulfide bonds with opposing Cxs on the adjacent cell and together they form conduits between cells by forming an impenetrable β-barrel structure (Foote et al., 1998). These cysteines appear in a highly conserved sequence: E1: C-X6-C-X3-C and E2: C-X5-C-X5-C and the degree of sequence conservation between Cx types allows heterogeneous GJs to form (Cottrell and Burt, 2005). The formation of heterogeneous GJs may serve to increase variability in channel permeability and conductance due to combinations of Cx types expressed by the same cells (Zhang and Nicholson, 1994).

Cx proteins have three cytoplasmic domains: a short amino-terminus (NT), a cytoplasmic loop (CL) and, of particular importance, some Cx proteins contain a long carboxyl-terminal tail (CT) that varies considerably in both sequence and length between Cx proteins (Figure 1.4). All three cytoplasmic domains are flexible, containing short α-helices separated by hinge domains (Sorgen et al., 2004). Regions of all three cytoplasmic domains have been implicated in channel-gating which may be regulated by a ball-and-chain method, by protein-interactions, or by sensitivity of these domains to voltage (Evans and Martin, 2002). Of the four TM domains, TM3 is the most amphipathic and was therefore predicted to line the aqueous channel (Zimmer et al., 1987). This has been confirmed by the Substituted Cysteine Accessibility Method (SCAM) and structural studies (Bogdanov et al., 2005).
**Figure 1.4 Structure of gap junction protein connexin 43.** Schematic tertiary structure of a single Cx43 protein in a lipid bilayer. Cylinders represent α-helices. Cytoplasmic domains are indicated in green (NT stands for the N-terminus, CL for the cytoplasmic loop and CT for the C-terminus), transmembrane domains (TM) in orange, and extracellular domains (E) in blue. Circles represent relevant amino acid residues, numbered according to their sequence from N- to C-terminus. Yellow circles represent tyrosine (Y) residues, red circles cysteine (C) residues and purple circles threonine (T) residues. The sequence of a proline (P)-rich sequence hypothesized to be a SH3 domain binding site is shown in grey. The position where the CT (ΔCT/246) domain is commonly truncated in Cx43 mutant constructs is indicated by the red line. (Not drawn to scale).
The Cx genes are divided into three subgroups (α, β, and γ) based on sequence identity and tail length (for review see (Söhl and Willecke, 2004)). Most have two exons flanked by a 5’- and a 3’-untranslated region (UTR) and separated by a single intron of varying length. The 3’-most exon contains the coding region. Knockout mice have been made for 13 of the 20 Cx types resulting in a range of phenotypes. In addition, human diseases such as oculodentodigital dysplasia (ODDD), Charcot-Marie tooth disease, non-syndromic hereditary deafness, and erythrokeratodermis variabilis have been linked to mutations in Cx43, Cx32, Cx26, and Cx31 genes and specific regions of the proteins have been linked with the disease state (Paznekas et al., 2009).

Cx proteins are co-translationally inserted into the endoplasmic reticulum (ER) where inter-loop disulfide bonds form between E1 and E2 and where miss-foldings are recognized by resident chaperone proteins. Chaperone proteins such as ERp29 have also been shown to prevent oligomerization from occurring prematurely in the ER (Das et al., 2009). Oligomerization into six-membered rings called “hemichannels” (HCs) or “connexons” occurs in the trans-golgi prior to trafficking to the plasma membrane. Microtubule-binding and caveolin are required for transport to the plasma membrane and possibly into lipid raft domains. Delivery to the plasma membrane is followed by lateral diffusion of HCs and incorporation into the periphery of existing GJ plaques (Lauf et al., 2002).

HCs are transported to the plasma membrane in their closed conformation and open upon GJ docking (Bukauskas et al., 1995), although increasing evidence shows that HCs may open under certain conditions and exchange molecules between the cytoplasm and the
extracellular space (for review see (Evans et al., 2006)). Cx HCs were proposed after a large increase in conductance was noticed in cells that had been caused to overexpress Cx40 (Bukauskas et al., 1995). While HCs have now been measured for many Cx types, the physiological relevance of Cx HCs is still debated. HCs may play a role in autocrine/paracrine signaling by allowing the release of adenosine triphosphate (ATP) to locally activate purinergic receptors (Baroja-Mazo et al., 2012).

GJ plaque size and location are determined by scaffolding proteins that bind to cytoplasmic regions of Cx proteins and link them to the cytoskeleton. Cx proteins have a relatively short half-life for junction proteins, being turned over every few hours. Old GJs are removed from the plaque interior whole in double-membrane structures called “annular junctions” or “connexosomes” which fuse with lysozymes for degradation. Cxs are targeted for internalization and degradation by phosphorylation, as well as mono- and poly-ubiquitination (Solan and Lampe, 2009b).

Although GJs are much less voltage-sensitive than other ion channels, they can be regulated by changes in potential across the GJ connecting two cell membranes (Vj) or across a single membrane (Vm) in the case of HC gating. GJ channels and HCs are also gated by pH, concentration of divalent cations (especially Ca$^{2+}$), and phosphorylation. Phosphorylation is likely the most relevant method of gating GJ channels in physiological settings (Solan and Lampe, 2009b) (Chapter 1.4.1) although other methods of channel-gating have been important for studies of GJ and HC properties and for the identification of residues or protein domains relevant to gating of these channels.
1.3.2 The role of connexins in the immune response

Connexins are expressed by immune cells themselves, as well as cell types found in immune cell niches such as lymph nodes, bone marrow, endothelial cells, and the thymus (Alves et al., 2005; Dorshkind et al., 1993; Krenacs and Rosendaal, 1995; Larson et al., 1997). B-lymphocytes have been shown to express Cx40 and Cx43 at both the protein and the messenger RNA (mRNA) level, and not to express Cx26, Cx32, Cx37, or Cx45 (Oviedo-orta et al., 2008), although expression has not be tested for all 20 Cx proteins. GJs are important for normal hematopoiesis by facilitating communication between bone marrow and stromal cells, and likely between stromal cells and developing blood cells (Montecino-Rodriguez and Dorshkind, 2001). Cx43−/− mice are postnatal lethal making them an inefficient tool for studying the role of Cx43 in the immune response, however heterozygous Cx43+/− mice have reduced numbers of T- and B-cells as measured using markers IgM and CD19 for B-cells and CD4 and CD3 for T-cells (Montecino-Rodriguez et al., 2000). Therefore Cx43 likely plays a role in development of lymphocytes at the least, although the importance of Cxs is likely undervalued since other Cx types probably compensate in individual Cx-knockdown experiments.

Cx43 expression is up regulated at both the mRNA and protein level in response to lipopolysaccharide (LPS)-induced inflammation in leukocytes and endothelial cells both in vitro and in vivo (Jara et al., 1995), as well as in macrophages (Eugenín et al., 2003). Inflammation caused by lysozyme injection into mouse footpad caused increased expression
of Cx43 in lymph nodes and particularly in the light zone of germinal centers where B cell fate decisions are made (Krenacs et al., 2005), suggesting that Cx43 may play a role in germinal center organization and B-cell differentiation.

GJ coupling in lymphocytes was first demonstrated in 1974 by microelectrode studies of isolated human peripheral blood lymphocytes (Oliveira-Castro and Barcinski, 1974). GJ coupling has been reported between B-cells and T-cells, with other B-cells (Oviedo - orta et al., 2008), and with follicular dendritic cells (FDCs) (Krenacs et al., 2005). Blocking GJ channel function in mixed T- and B-lymphocytes cultures with drugs or mimetic peptides reduced Ig and cytokine secretion in vitro (Oveido-Orta et al., 2001).

While GJ intracellular communication (GJIC) is difficult to study in leukocytes due to their highly migratory behavior and formation of relatively transient cell-cell contacts, increasing evidence suggests that HCs may play a role in various immune responses. ATP release through HCs is emerging as a mechanism for signal amplification and has been implicated in neutrophil and macrophage chemotaxis (Chen et al., 2006), T-cell activation (Mendoza-Naranjo et al., 2011), and macrophage-induced cell killing. ATP released through HCs can act as an autocrine and paracrine signal, stimulating purinergic receptors on nearby cells. Purinergic receptor signaling leads to opening of both Panx and Cx HCs and to Ca\(^{2+}\) influx (for review see (Baroja-Mazo et al., 2012)). While the effects of HC activity have mostly been attributed to Panxs, which exclusively form HCs (Bruzone et al., 2003), the increasing evidence for Cx HCs should make us aware that Cx HCs may play a role in these responses as well.
Communication between B-cells and other immune cell types is essential for cross-presentation and co-stimulation, though whether Cxs play a role in these responses has yet to be studied. A role for GJs in cross-presentation of Ag from infected cells to professional APCs like dendritic cells has been hypothesized by experiments showing the direct transfer of small linear peptides between cells expressing Cx43 and exhibiting GJIC (Neijssen et al., 2005). These peptides were subsequently presented on MHC I molecules and lead to cytotoxic T-cell activation as measured by interferon-γ (IFN-γ) production. B cells are professional antigen presenting cells (APCs) therefore it may be expected that Cx43 may contribute to Ag transfer and presentation in these cells as well. For a review of GJ mediated Ag-presentation see (Handel et al., 2007).

1.3.3 Non-channel functions of connexins

Over the past decade, a number of studies have shown that Cxs have effects independent of GJ channel function. These studies have taken advantage of pharmacological agents to block channels (Guan et al., 1996; Silverman et al., 2008), mimetic peptides (Leybaert et al., 2003) and antibody neutralization of specific regions of the Cx protein (Hofer and Dermietzel, 1998), as well as mutations known to block channel function (Beahm et al., 2006) or to prevent trafficking to the cell surface where GJs are able to form (Tong et al., 2007). While some effects of Cx expression may be attributed to HC activity rather than GJ formation, many effects are independent of channel activity altogether. Channel-blocking studies suggest an
alternate mechanism of Cx’s action such as protein-protein interactions involving cytoplasmic regions like the CT that could participate in scaffolding or propagation of signaling cascades.

Two functions of Cxs are frequently reported to be independent of GJ function: firstly the role of Cxs on cell cycle progression and viability, and secondly, on cell motility and migration. Initially the effect of Cxs on cell growth was presumed to be a by-stander effect where GJs facilitate the diffusion of pro-survival or pro-death signals to neighboring cells (Mesnil and Yamasaki, 2000). However many techniques have been used to demonstrate that growth effects are independent of GJ communication. As an example Cx37 slows cell cycle progression in rat insulinoma cells (Burt et al., 2008), and this effect is reproducible with Cx43 mutant T154A which does not form functional channels (Good et al., 2011). A pair of Cx26 mutants that either blocked channel function, or both trafficking to the plasma membrane and channel function still caused reversion of tumor phenotype in breast cancer cells (Kalra et al., 2006).

Glioma cells expressing Cx43 mutant C61S were protected from apoptosis even though this mutation prevented proper trafficking to the plasma membrane and therefore channel formation (Lin, Yang et al. 2003). Similarly, the Cx43 CT alone is sufficient to suppress growth of HeLa cells even though this peptide remains cytoplasmic and can not form GJs (Dang et al., 2003). Cx43 regulates growth independently of GJ formation since serine point-mutants block growth effects of Cx43 but not channel formation in N2A cells (Moorby and Patel, 2001), indicating that serine residues in the CT region of Cx43 may not only regulate GJ turnover, but also participate in signaling pathways leading to regulation of cell growth.
Interestingly, Cx43 overexpressing glioma cells also exhibited cytoskeletal effects, namely flattening of cells and stress-fiber formation, and these reorganizations of the cytoskeleton were actually required for Cx43’s protective effect against apoptosis (Lin, Yang et al. 2003). Cytoskeletal changes are often noted with up- or down-regulation of Cx43 which leads us to the next effect of Cxs that is often channel-independent: cellular motility and migration. This discussion will focus exclusively on Cx43 which has been the subject of the most Cx research and is particularly well-studied for its role in cell motility and migration.

1.4 Connexin 43

1.4.1 Regulation of Cx43 through phosphorylation

Cx43 is the most widely expressed Cx protein, having been detected in over 40 different cell types and tissues (Oyamada et al., 2005). Cx43 was first isolated from myocardial GJs in 1980 (Kensler and Goodenough, 1980) and sequenced in 1987 (Nicholson et al., 1987). Cx43 has one of the longest CT tails of the Cx proteins (155 amino acids long), containing multiple sites for protein interactions and regulation by phosphorylation.

Different forms of Cx43 can be separated based on mobility by gel-electrophoresis commonly resulting in two phosphatase-sensitive and one insensitive band. The non-phosphorylated form of Cx43 (P0) runs at approximately 42 kDa, P1 at 44 kDa and P2 which is the form most commonly associated with GJ plaques at 46 kDa (Solan and Lampe, 2009a). These mobility
shifts are likely the result of conformational changes to the Cx43 protein caused by phosphorylation of structurally important aa side chains or due to altered protein-interactions affecting Cx43 conformation, since the addition of a single inorganic phosphate group is not sufficient to account for a 2 kDa shift in molecular weight (Solan and Lampe, 2009a).

The CT domain of Cx43 is the target of phosphorylation and contains multiple serine, threonine, and tyrosine residues. Cx43 phosphorylation has been shown to regulate protein trafficking, assembly and disassembly of GJ plaques, internalization, and channel gating. Some examples of kinases that have been shown to phosphorylate Cx43 include serine kinases such as protein kinase A (PKA) which affects Cx43 trafficking, protein kinase C (PKC) which influences GJ channel conductance and cell-cycle progression, casein kinase 1 (CK1) which is involved in assembly of Cxs into GJ plaques, and mitogen-activated protein kinase (MAPK) which causes GJ internalization. The tyrosine kinases v-Src and c-Src can also decrease GJ conductance by phosphorylation of serine residues (Lampe and Lau, 2004).

In addition to phosphorylation sites, the Cx43 CT domain contains binding sites for protein-protein interactions. A proline rich domain located between amino acids (aa) 274-283 may bind SH3-domain containing proteins such as Src kinases. This motif has been hypothesized to bind v-Src, bringing it into sufficient proximity with Cx43 to facilitate the phosphorylation of Y265 and Y247 (Lin et al., 2001a). The CT also has a microtubule-binding domain (aa 234-243) that is necessary for trafficking of Cx43 to the plasma membrane (Shaw et al., 2007) and cell polarity (Francis et al., 2011a). Cx43 also contains a PDZ-binding domain near its
carboxyl-terminus (aa 379-382) which can bind the scaffolding protein zona occludens-1 (ZO-1) which anchors Cx43 to the cytoskeleton and stabilizes GJ plaques (Palatinus et al., 2011).

1.4.2 The role of Cx43 in cell migration

Cx43−/− mice die a few hours after birth from cardiac failure resulting from outflow tract obstructions and malformations of the right ventricle (Reaume et al., 1995). These malformations arise unexpectedly, not because of a defect in cardiac communication, but because of reduced cardiac neural crest cell migration from the somites to the outflow tract during embryogenesis (for review see (Lo et al., 1999)). Experiments on neural crest cells in vitro have shown that Cx43−/− cells are less effective at directional migration due to an inability to polarize and form a leading edge due to altered cytoskeletal organization (Xu et al., 2006). Cx43 has since been shown to influence polarization and motility of mouse embryonic fibroblasts (MEFs) isolated from Cx43−/− mice as well (Francis et al., 2011a) and this effect is channel-independent since the defect can be mimicked by forced expression of a dominant negative (DN) Cx43 mutant with a truncated CT tail in Cx43+/+ MEFs, but not with Cx43 mutant Y17S which does not form functional channels (Francis et al., 2011a). Increasingly, evidence shows a non-channel role of Cx43 in motility of many cell types.

While knockout mice make invaluable tools for looking at the role of individual genes, high throughput screens can identify many possible genes responsible for regulating a common phenotype. Cx43 was identified in an unbiased small interfering RNA (siRNA) knockdown
screen for genes involved in migration using an \textit{in vitro} epithelial wound-healing assay (Simpson et al., 2008). Cx43 expression was shown to enhance migration of Cx43-null HeLa cells independently of channel function since the CT alone which doesn’t get expressed at the plasma membrane or make channels was sufficient to enhance migration, yet the NT capable of forming channels was not sufficient (Behrens et al., 2010).

The role of Cx43 on cell migration has been well characterized in neural cell types. Neuronal migration along radial glia in the developing mouse neocortex is enhanced by Cx43 and is not blocked by mutant Cx43T154A which does not make channels (Elias et al., 2007). In addition to its role during normal development, Cx43 can enhance tumor cell migration. Gliomas are highly invasive astrocyte tumors, whose invasiveness is enhanced by Cx43 expression (Lin et al., 2002; Zhang et al., 2003). Channel blocking drug carbenoxolone (CBX) did not block glioma migration \textit{in vitro} indicating that this is a channel-independent phenomenon (Bates et al., 2007). Since Cxs have largely been considered tumor suppressors, recognition of their potential to increase tumor invasiveness is an important consideration for understanding the cell biology of tumor cells and for the development of possible anti-cancer therapeutics.

GJs also form between lymphocytes and endothelial cells during transmigration, suggesting a role for Cxs in lymphocyte migration (Oviedo-Orta et al., 2002). Transmigration occurs when lymphocytes cross the endothelium lining post-capillary venules and exit into the interstitial tissue in response to inflammatory cues. Signaling cross-talk between endothelial cells and lymphocytes during this process facilitate adhesion and shape changes to the lymphocyte (accomplished by reorganization of the actin cytoskeleton) in order for these cells to squeeze
through gaps between endothelial cells. In experiments by Oveido-Orta et al. dye transfer could be blocked using mimetic peptides against Cx but transmigration occurred normally, suggesting that migration is independent of GJ channel function (Oviedo-Orta et al., 2002).

1.5 The role of Cx43 in B-lymphocytes

1.5.1 Cx43 in BCR-mediated spreading

BCR signaling in the immature B cell line Wehi231 stimulated with anti-IgM resulted in a time-dependent shift in Cx43 molecular weight by gel electrophoresis. The resulting Cx43 band-shift was phosphatase-sensitive, and therefore probably due to phosphorylation (Bruzzone et al., 2003; Machtaler et al., 2011). Cx43 has a long cytoplasmic CT tail with potential phosphorylation sites. Phosphorylation of tyrosine and serine residues of the Cx43 CT have been implicated in regulation of Cx43 turnover, channel conductance, and cell growth, leading us to investigate this protein in B-cells further.

Cx43 is involved in BCR-mediated spreading as shown by both loss-of-function (LOF) and gain-of-function (GOF) approaches. Knockdown of Cx43 by siRNA in the Wehi231 cell line resulted in reduced spreading on anti-IgM coated glass, whereas expression of EGFP tagged Cx43 in a Cx43-null plasmacytoma cell line that had previously been transfected with the BCR (J558µm3) caused these cells to spread. J558µm3 cells usually fail to spread on anti-IgM coated glass, merely extending and retracting small membrane protuberances on their upper surface (Bruzzone et al., 2003; Machtaler et al., 2011).
BCR-mediated spreading of the mature B-cell line A20 requires the activation of the Rap1 GTPase (Lin et al., 2008a), therefore B-cells expressing different levels of Cx43 were assessed for Rap1 activity. While Rap1-GTP was detected initially after BCR stimulation, lower levels of active Rap1 were detected in Wehi231 cells expressing siRNA against Cx43. Additionally, sustained activation of Rap1 was detected in J558µm3 cells expressing EGFP fused Cx43 but only transient Rap1 activation was induced by BCR stimulation of J558µm3 cells expressing EGFP alone (Bruzzone et al., 2003; Machtaler et al., 2011).

A Cx43 mutant with the CT domain truncated immediately after the microtubule-binding site (Δ246) has been used previously to examine the role of the Cx43 tail and has been shown to have normal trafficking to the plasma membrane (Bates et al., 2007). Expression of this mutant in J558µm3 cells was not sufficient to cause rapid, radial BCR-mediated spreading or Rap1 activation, demonstrating the importance of the Cx43 CT domain in BCR-mediated spreading response (Bruzzone et al., 2003; Machtaler et al., 2011). While these experiments demonstrate the importance of the CT domain, the mechanism underlying these effects remains unknown. The requirement of Cx43 for sustained Rap1 activation suggests a role for the CT domain in propagating BCR signaling, but other possibilities include a scaffolding role where Cx43 stabilizes the actin cytoskeleton through protein-interactions with its CT domain, or channel gating by the CT tail.
1.5.2 Cx43 in B-cell adhesion and migration

Since Cx43 expression was found to improve activation of the Rap1 GTPase, the influence of Cx43 was investigated in other processes that are controlled by Rap1. Rap1 also regulates B-cell adhesion to endothelial cell layers and transmigration (McLeod et al., 2002a; McLeod et al., 2004). The effects of Rap1 on adhesion and migration are also relevant in the case of lymphoma invasion as shown by competitive homing experiments in mice (Lin et al., 2009).

Cx43 expression caused more sustained Rap1 activation in response to integrin engagement by anti-lymphocyte associated antigen 1 (LFA-1) as well as stimulation with chemokine CXCL12/SDF-1. Cx43 knockdown impaired Wehi231 adhesion to an endothelial monolayer as well as motility and transmigration of Wehi231 cells towards a chemokine gradient (Bruzzone et al., 2003; Machtaler et al., 2011). These results are consistent with the role of Cx43 in enhanced migration of other cell types.

1.6 Purpose of thesis study

B-cell adhesion, spreading, and migration are influenced by expression of the protein Cx43 by a mechanism involving the CT domain, yet the exact mechanism by which the CT is involved remains unclear. Mutational studies of Cx43 in neural migration have returned conflicting results regarding the mechanism of Cx43’s influence. The CT domain of Cx43 has been implicated in glioma and neuronal migration since CT-truncation of Cx43 destroys its
migration-enhancing effects (Bates et al., 2007; Gina et al., 2009). Yet glioma migration was unaffected by treatment with channel-blocking drug carbenoxolone (CBX) (Bates et al., 2007), so it is unlikely that migration is affected by the ability of the CT to regulate GJ channel conductance. The CT domain presumably influences glioma migration by acting as a scaffold for protein-protein interactions involved in either signaling cascades or by anchoring cytoskeletal components to the plasma membrane.

An alternative hypothesis that comes from experiments in vivo is that the extracellular domains of Cx43 may provide dynamic adhesive contacts by GJ formation between the migrating cell and the cellular substratum. Experiments using antibodies against the extracellular domains of Cx43 to block GJ adhesion showed that blocking GJ formation prevented neuronal migration, as did expression of mutant C61S which are unable to form the disulfide bonds required for GJ docking (Elias et al., 2007). Expression of channel-mutant Cx43T154A had no affect on neuronal migration, so both studies agree that Cx43’s affect on neural migration is channel-independent.

While Cx43’s effect on neural migration appears to be channel-independent, it is important to rule-out the possibility that Cx43 influences adhesion, spreading, and migration through channel formation in B-cells since channel formation is the canonical function of GJ proteins. The function of Cx43 in forming HCs has been unexplored in B-lymphocytes. Increasing evidence for HC function in communication in the immune system (Junger, 2011) makes addressing the possible involvement of Cx HCs in B-cell processes an important area of study.
Given the role of Cx43 in B-cell adhesion, spreading and migration, the goals of this project have been to investigate the contribution of different domains of Cx43 to B-cell cytoskeletal rearrangements. This project investigated the contributions of different domains of Cx43 in more detail. Specifically, we investigated the role of cysteine residues in the extracellular loops, a threonine residue lining the channel, and a potential tyrosine phosphorylation site in the tail of Cx43. This was done by using a gain-of-function approach by expressing a panel of Cx43 mutants in a tissue culture B-cell line that does not express endogenous Cx43: the plasmacytoma cell line J558μm3. The different transfected cells were tested using a BCR-mediated B-cell spreading assay as a readout for B-cell processes that are dependent on cytoskeletal rearrangements. These studies will help to define the important regions of Cx43 that affect B-cell spreading, as well as identify which region we should focus on for future studies. These results will be useful in understanding the potential role of the motifs of Cx43 in normal B-cell functions important for the immune response.

**Overarching aim:**

To identify the functional domains of Cx43 (extracellular, channel-lining, or cytoplasmic) that contribute to its importance in BCR-mediated B-cell spreading through mutational studies of the Cx43 protein by targeting specific amino acids for mutation.
Hypotheses:

1) The effect of Cx43 on BCR-mediated B-cell spreading is independent of adhesion mediated through the formation of GJs between cells.

2) The effect of Cx43 on BCR-mediated B-cell spreading is independent of channel activity.

3) The effect of Cx43 on BCR-mediated B-cell spreading depends on protein-protein interactions between Cx43 and effectors involved in BCR-signaling.

1.7 Summary of findings

1) The extracellular domains of Cx43 were targeted by mutation of the cysteine residues involved in GJ docking and we discovered that substitution of these residues drastically altered the ability of the protein to traffic to the cell surface. After initial characterization of the mutated Cx43 proteins expressed in J558μm3, they were not pursued. The data obtained appears in the Appendix.

2) The TM region was examined by using the Cx43T154A mutant, which blocks Cx43 channel function (Beahm et al., 2006). To assess this a dye-uptake assay of HC activity adapted for B-cells showed that Cx43 expressing B-cell lines A20 and Wehi231 fail to form functional HCs upon stimulation by the common HC activator: removal of divalent cations. Consistent with other overexpression studies, a Cx43-null plasmacytoma B-cell line (J558μm3) transduced
with EGFP fused Cx43 showed some dye-uptake even in the presence of Ca\(^{2+}/\)Mg\(^{2+}\) containing medium. This uptake was not seen in J558µm3 expressing EGFP alone, and was selectively blocked by CBX, showing that dye-uptake occurred through Cx43HCs. While Cx overexpression was sufficient to lead to some HC activity in B-cells, these channels probably do not account for the effect of Cx43 expression on BCR-mediated B-cell spreading since treatment with HC blocking drugs carbenoxolone (CBX), probenecid (Pbn), and lanthanum (La) had no effect on B-cell spreading of A20, Wehi231, J558µm3+Cx43-EGFP, or primary splenic B-cells isolated from mice. These results suggest that Cx43 influences BCR-mediated B cell spreading by non-channel mechanisms, perhaps by acting as a scaffolding for protein interactions through binding sites found in the Cx43 cytoplasmic CT domain.

In support of this idea, we found that point mutant Cx43T154A caused a non-radial spreading phenotype in J558µm3 cells characterized by the formation of actin and Cx43-rich projections when plated on anti-BCR coated glass coverslips. This phenotype was ablated by truncation of the CT tail (Δ246), indicating that this phenotype was not exclusively the product of reduced channel-conductance, but depended upon the CT domain, which is consistent with our lab’s previous finding that the CT domain of Cx43 is necessary for BCR-mediated B-cell spreading.

We have previously found that Cx43 expression improves the sustained activation of the Rap1 GTPase in response to BCR stimulation using a biochemical assay of Rap1 activation. In this study, we further demonstrated that Cx43 expression leads to BCR-mediated J558µm3 cell spreading through the activation of Rap1 because forced expression of a Rap1-specific
GAP (RapGAPII) which converts Rap1 into its inactive, GDP-bound state, was sufficient to prevent spreading by J558µm3+Cx43-EGFP cells. Conversely, expression of a constitutively active form of Rap1 (Rap1V12) by J558µm3 caused cell spreading. Unlike expression of WT Cx43, the expression of Cx43T154A and Cx43Δ246 do not result in sustained Rap1 activation following BCR stimulation, suggesting that both mutants influence Rap1 activation through a similar mechanism. We hypothesize that point mutation of T154 may result in a conformational defect that specifically affects the Cx43 CT domain, in addition to its well-characterized effect on GJ channel conductance.

3) To further investigate the role of the Cx43 CT domain, point mutants of a putative Src-kinase binding site were made by site-directed-mutagenesis Y265F and Y265D. Tyrosine 265 has been hypothesized to be both phosphorylated and then bound by Src-kinase through its SH2 domain for subsequent phosphorylation of tyrosine 247 (Lin et al., 2001a). Both point mutants of Y265 were sufficient to block BCR-mediated B-cell spreading in J558µm3 cells, highlighting the importance of a single residue for Cx43’s influence on cell spreading.

BCR stimulation results in a phosphatase-sensitive shift of Cx43 mobility by gel electrophoresis (Bruzzone et al., 2003; Machtaler et al., 2011). J558µm3 cells expressing Cx43Y265F and Cx43Y265D exhibit a comparable shift to WT Cx43 in response to BCR-stimulation, which suggests that Cx43 is phosphorylated at other residues besides Y265, in spite of the importance of this residue in BCR-mediated spreading. In fact, BCR stimulation does not appear to result in tyrosine phosphorylation of Cx43 as shown biochemically by immunoprecipitation (IP) of either endogenous Cx43 from Wehi231 or overexpressed Cx43-
EGFP from J558um3 followed by detection with a phospho-tyrosine antibody (pY). The Cx43 CT domain contains a far greater number of serine residues than tyrosine residues and these may be targets of phosphorylation as well.

Our lab’s on-going goals are to determine the type of phosphorylation responsible for Cx43 band-shift in response to BCR-stimulation as well as to further uncover the mechanism explaining the importance of the CT domain of Cx43 to BCR-mediated B-cell spreading. Because of the expansive nature that a phosphorylation study would entail, this part was considered by us and by the supervisory committee as being beyond the scope of the MSc thesis and that the role of phosphorylation of the CT tail will be carried out by MSc student Farnaz Pournia who is using a series of Cx43 truncations to determine the functionally important regions of the CT domain.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials and reagents

2.1.1 Plasmids

The AP2 and NAP2 expression vectors were gifts from Dr. Christian Naus (Dept. Cellular and Physiological Sciences, UBC). AP2 is a bicistronic murine retroviral vector containing a multiple cloning site for insertion, under a CMV promoter and EGFP under an IRES promoter (Mao et al., 2000). The IRES and EGFP were removed from NAP2 for expression of EGFP-fusion constructs (Galipeau et al., 1999). NAP2 containing cDNA encoding Wild-Type (WT) Cx43 with EGFP fused to the C-terminal (CT) tail in-frame (Cx43-EGFP) as well as mutated Cx43 with a truncated CT tail (Cx43Δ246-EGFP) have been described previously (Bates et al., 2007).

Additional plasmids containing mutated Cx43 cDNA were created by performing site-directed mutagenesis (Section 2.2.6) on the above-mentioned WT or truncated Cx43 constructs by using custom designed primer pairs (Table 2.1). Two tyrosine (Y) residues found in the Cx43 cytoplasmic CT tail have been described as putative Src-binding sites (Lin et al., 2001a). The first of these, Y265 was substituted with either phenylalanine (Y265F) or aspartic acid (Y265D) using site-directed mutagenesis (see Section 2.2.6) performed on WT Cx43-EGFP in the NAP2 expression vector.
The channel-blocked mutant Cx43T154A was generated using previously published primers (Beahm et al., 2006). Site-directed mutagenesis was performed on both WT Cx43-EGFP and Cx43Δ246-EGFP, both in the NAP2 expression vector.

To study the role of the Cx43 extracellular loops, and specifically at disulfide bonding, successive cysteine residues were substituted with alanine using WT Cx43-EGFP in the NAP2 vector. Initially a single primer pair containing three substitutions was used for each loop. However, due to the spacing between residues, the most 5’ substitution was found to be too close to the end of the primer and was not incorporated into the final sequence. An additional two primer pairs were designed to mutate the most 5’ cysteine residue of each loop, resulting in one mutant lacking all six cysteine residues (CL6) and three intermediates (CL2, CL3, CL5). Mutants were named for the number of cysteine residues substituted with alanine.
Table 2.1 Primer sets custom ordered for site directed mutagenesis. Primers were ordered through integrated DNA technologies (Integrated DNA Technologies, Coralville Iowa).

<table>
<thead>
<tr>
<th>Resulting mutation</th>
<th>Primer names</th>
<th>Primer sequence (5’-3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Cx43</td>
<td>Cx43 f</td>
<td>ATGGGTGACTGGAGTGCCCTG6G6</td>
<td>Binds coding strand of Cx43 for sequencing</td>
</tr>
<tr>
<td></td>
<td>Cx43 r</td>
<td>AATCTCCAGGTCATCAGGGCCAGG</td>
<td></td>
</tr>
<tr>
<td>Y265F</td>
<td>Y265F f</td>
<td>GCGGATCTCCAAATTGGCCCTACTTC</td>
<td>Binds Cx43 template, exchanges tyrosine 265 for phenylalanine</td>
</tr>
<tr>
<td></td>
<td>Y265F r</td>
<td>GCAAGCATTGAGTAGGGGATTTTG</td>
<td></td>
</tr>
<tr>
<td>Y265D</td>
<td>Y265D f</td>
<td>GCGGATCTCCAAAGAGGCTACTTC</td>
<td>Binds Cx43 template, exchanges tyrosine 265 for aspartic acid</td>
</tr>
<tr>
<td></td>
<td>Y265D r</td>
<td>GCAAGCATTGAGTAGGGGCTTTTG</td>
<td></td>
</tr>
<tr>
<td>T154A</td>
<td>T154A f</td>
<td>GGCAGGTTCATGAGGTCAGCATCAT</td>
<td>Binds Cx43 template, exchanges threonine 154 for alanine</td>
</tr>
<tr>
<td></td>
<td>T154A r</td>
<td>GGAATGCTGATGAATAGGGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>T154AΔ246</td>
<td>T154A f</td>
<td>GGCAGGTTCATGAGGCTACATCAT</td>
<td>Binds Cx43ΔCT template, exchanges threonine 154 for alanine</td>
</tr>
<tr>
<td></td>
<td>T154A r</td>
<td>GGAATGCTGATGAATAGGGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>CL2</td>
<td>C54,61,65A f</td>
<td>GCGGCTAACACTCAACAACCTGGCCGC</td>
<td>Binds to Cx43 template, exchanges cysteines 61 and 65 for alanine (C54 was too close to 5’ end)</td>
</tr>
<tr>
<td></td>
<td>C54,61,65A r</td>
<td>GTCATAGGCCAGGCTTTTCCGGCCAA</td>
<td></td>
</tr>
<tr>
<td>CL3</td>
<td>C54A f</td>
<td>GGTTGATGACAGTCTGCTTCTCGGC</td>
<td>Binds to Cx43CL2 template, exchanges cysteine 54 for alanine</td>
</tr>
<tr>
<td></td>
<td>C54A r</td>
<td>GCGAGGTGTGTAGTGAGTGCGAGA</td>
<td></td>
</tr>
<tr>
<td>CL5</td>
<td>C187,192,198A f</td>
<td>CCGCCAAGAGAGATACTCGCGCGC</td>
<td>Binds to Cx43CL3 template, exchanges cysteines 192 and 198 for alanine (C187 was too close to 5’ end)</td>
</tr>
<tr>
<td></td>
<td>C187,192,198A r</td>
<td>GAGAAGCCGCTACACTTGGTGGC</td>
<td></td>
</tr>
<tr>
<td>CL6</td>
<td>C187A f</td>
<td>GCGCGGTCTACAGCGGCTAGAGAGA</td>
<td>Binds to Cx43CL5 template, exchanges cysteine 187 for alanine</td>
</tr>
<tr>
<td></td>
<td>C187A r</td>
<td>CGCGGGATCTCTCTGGCGGGTGTAG</td>
<td></td>
</tr>
</tbody>
</table>
2.1.2 Antibodies

The polyclonal goat α-mouse IgM used for cell stimulation (Section 2.3.3), immunoblotting (Section 2.4.2), staining (Section 2.7.1), and for coating glass coverslips for cell spreading assays (Section 2.6.1), was purchased from Jackson Immuno Research Laboratories (West Grove, Pennsylvania, #115-005-020) and was specific to the μ heavy chain. For staining surface membrane IgM (mIgM) and analysis by flow-cytometry (Section 2.5.1), monoclonal phycoerythrin (PE)-conjugated rat α-mouse IgM was purchased from eBiosciences (San Diego, California, #12-5790-81).

For immunoblotting, the polyclonal rabbit α-mouse antibody against the CT tail of Cx43 was purchased from Sigma-Aldrich (Oakville, Ontario, #C6219). The polyclonal mouse α-mouse antibody against the N-terminal region of Cx43 was purchased from the Fred Hutchinson Cancer Research Institute (Seattle, Washington). The monoclonal rabbit α-mouse antibody specific for Cx43 phosphorylated at Y265 was obtained from Dr. Paul D. Lampe (Fred Hutchinson Cancer Research Institute) (Solan and Lampe, 2008). Polyclonal rabbit α-mouse Akt and rabbit α-mouse Rap1 were purchased from Cell Signaling Technologies (Santa Cruz, California, #9272 and #23995 respectively). The polyclonal mouse α-mouse antibody against actin was purchased from Fisher Scientific (Fair Lawn, New Jersey, #ICN691001).

The monoclonal mouse antibody capable of binding all species of phospho-tyrosine (pY) was prepared in-house as has been described previously (Richards et al., 1996). The 4G10 hybridoma cells (Morrison et al., 1989) were grown to confluency, the cells left to die to
promote concentrated antibody in the growth medium, and the supernatants containing monoclonal antibodies were filter sterilized, aliquoted and stored at -20°C.

Secondary antibodies were conjugated to horseradish peroxidase (HRP) enzyme for detection with chemiluminescence. Polyclonal goat α-mouse and goat α-rabbit IgG (κ chain specific) was purchased from Bio-Rad (Mississauga, Ontario, #170-6516 and #170-6515 respectively). Polyclonal donkey α-goat IgG (κ chain specific) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, #SC2020)(Table 2.3).

2.1.3 Cell lines

The J558µm3 B cell line which expresses a full 4 chain (IgM, Igα and Igβ) B cell antigen receptor (BCR) at the plasma membrane was a gift from Dr. Louis Justement (University of Alabama, Birmingham, (Justement et al., 1990)) and BOSC 23 retroviral packaging cell line was a gift from Dr. Warren S Pear (Massachusetts Institute of Technology, Cambridge (Pear et al., 1993)). Wehi231 and A20 B cells were obtained from the American Type Culture Collection (ATCC)(Rockville, Maryland). Primary murine B cells were isolated from Black-6 mice spleens by negative selection using the EasySep® Mouse B Cell Enrichment Kit from Stem Cell Technologies (Vancouver, British Columbia, #19754).
2.1.4 Mice

C57BL/6 mice were obtained by UBC Animal license and breeding program #A10-0384 and spleens were removed and B-cells prepared as described and approved as according to the Matsuuchi Lab animal license #A11-0317. Astrocyte cultures from both wild-type mice were obtained from 0-1 day old pups. Neocortices were dissected in PBS and placed in culture medium consisting of high glucose DMEM with 10% FBS. Tissue was triturated through a serological pipette and passed through a 70 μm cell strainer (BD Falcon, Bedford, MA). Cells were re-suspended in culture medium and plated on either plastic culture dishes or laminin coated coverslips and stored in a humidified incubator in 95% air/5% CO2 at 37°C. Medium changes were performed every 4 days and astrocytes were used between 14-21 days in vitro or when 30% confluent for the hemichannel experiments described below (~5 days).

2.2 Molecular biology techniques

2.2.1 Bacterial transformation

Transformation was carried out as per manufacturer’s instructions. Sub-cloning competent and ultra-competent strains of E.coli, DH5α and XL10-GOLD were purchased from Invitrogen (Burlington, Ontario, #18265017) and Stratagene (Agilent Technologies, Mississauga, Ontario, #200314) respectively. Briefly, DNA was added to bacteria which were incubated on ice for 30 min, heat shocked at 42°C for 30 sec, incubated an additional hour in 1 ml pre-warmed lysogeny broth (LB, also referred to as “Luria broth” or “Luria-Bertani” medium
(Bertani, 2004)) medium (10 g/L bacto-tryptone (BD Biosciences, Mississauga, Ontario, #211705), 5 g/L bacto-yeast extract (BD Biosciences, #212750), 10 g/L NaCl (Fisher, #BP358-212), pH 7) while shaking at 250 rpm and then plated on LB agar plates (LB media containing 20 g/L bacto-agar (BD Biosciences, #214010), autoclaved and poured into petri dishes) containing 100 µg/ml ampicillin (Amp) (Sigma, #A9518) for selection. Colonies were isolated from plates 18-36 hours after incubation at 37°C.

2.2.2 DNA preparation

A single bacterial colony was used to inoculate 8-200 ml of LB broth containing 100 µg/ml Amp for selection, and was incubated overnight in 14 mL round bottom polypropylene tubes (BD Falcon, Franklin Lakes, New Jersey, #352059) at 37°C shaking at 250 rpm on a Lab Line Orbit Environ-Shaker (Melrose Park, Illinois). DNA was purified from overnight bacteria cultures using the Sigma-Aldrich GeneElute Miniprep kit (#NA0160) or Invitrogen PureLink HiPure Maxiprep kit (#K210017) according to the manufacturer’s instructions. Briefly, bacteria were pelleted from overnight cultures by centrifugation at 3000 rpm for 20 min in an IEC Centra-8R centrifuge (International Equipment, Nashville, Tennessee), bacteria were lysed, and DNA was isolated by either passage through a column or precipitation and collection by centrifugation. Isolated DNA was re-suspended in distilled water and DNA concentration and purity was assessed by a Nanodrop 1000 spectrometer (Thermo Scientific, Nepean, Ontario).
2.2.3 Restriction endonuclease digestion

Restriction enzymes were purchased from New England BioLabs (Whitby, Ontario) and added to DNA according to manufacturers instructions for 1 h at 37°C. The resulting digested DNA was run by agarose gel electrophoresis for identification (see Section 2.2.5).

2.2.4 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, New Jersey, #27-9558-01) according to the manufacturer’s instructions. To each bead, 25 pmol of each primer and 5 µg template DNA was added and reactions were run at 95°C for 45 sec, 55°C for 2 min and 72°C for 2 min, for 35 cycles on a PTC-100 programmable thermal controller from MJ Research Inc. (Watertown, Massachusetts). The resulting amplified DNA was run by agarose gel electrophoresis for identification (see Section 2.2.5).

2.2.5 Agarose gel electrophoresis

Agarose (Invitrogen, #15510-027) was dissolved to 1% in Tris-buffered ethylene diamine tetraacetic acid (EDTA: 90 mM Tris-HCl (Fisher, #BP152-5) pH 8.2, 90 mM boric acid (Fisher, #A73-500)), boiled and poured into a Horizon 58 horizontal gel electrophoresis apparatus (Gibco Life Technologies, Invitrogen) with 0.1% SYBERsafe (Molecular Probes, Invitrogen, #533102) for labeling DNA. Whole undigested plasmid, restriction endonuclease digested
(see Section 2.1.3), or PCR-amplified (see Section 2.1.4) DNA samples were mixed with an appropriate volume of DNA sample buffer (0.04% bromophenol blue (Bio-Rad, #161-0404), 0.04% xylene cyanol, 10% sucrose (Fisher, #BP220-212)) for loading, and the 1 kb Plus DNA ladder (Invitrogen, #10787-018) was used as a size standard. DNA was run at 100 V for approximately 1 h and imaged with an Alpha Imager EC MultiImage Light Cabinet (Alpha Innotech, San Leandro, California) using Alpha Imager software (Alpha Innotech).

2.2.6 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene, #210518) according to the manufacturer’s instructions. PCR was run at 95°C for 20 sec, 60°C for 20 sec, and 68°C for 3 min and 45 sec for 17 cycles using primers (Table 2.1) custom ordered through integrated DNA technologies (Integrated DNA Technologies, Coralville, Iowa). Resulting DNA was either run by agarose gel electrophoresis (see Section 2.1.5) to confirm amplification by PCR or purified from transformed XL-GOLD super-competent cells (see Sections 2.2.1 and 2.2.2). The UBC DNA sequencing laboratory, (NAPS, Vancouver, British Columbia; www.msl.ubc.ca/services/naps) was used to sequence plasmid DNA in order to check for the presence of the desired mutations by using primers flanking the coding sequence of wild-type (WT) Cx43 (Table 2.1).
2.3 Tissue culture

2.3.1 Cell culture

Suspension cells were maintained in 10 cm polystyrene tissue culture dishes (BD Falcon, #353003) in 10 ml of Roswell Park Memorial Institute (RPMI) medium (Gibco, Invitrogen, 21870-076) supplemented with 10% Fetal bovine serum (FBS) (Bio-Rad, #161-0404), 4.5 g/L glucose (Fisher, #D16-500), 2 mM L-glutamine (Sigma, #C8540), 1 mM sodium pyruvate (Sigma, #P5280), 50 µg/ml penicillin-streptomycin (Invitrogen, #15140122), and 50 µM β-mercaptoethanol (Bio-Rad, #17-1317-01). Cells were passaged every 2-3 days as required to be kept at 1-10x10^5 cells/ml in an direct heat incubator (ThermoForma, Champaign, Illinois) in an environment of 37°C with 5% CO₂. Suspension cells were passaged by centrifugation at 1,500 rpm for 5 min in an IEC Centra-CL3R refrigerated centrifuge (Thermo Electron Corporation, Gormley, Ontario) and re-suspension in fresh media. Adherent cells were incubated 1 min in trypsin (Gibco, Invitrogen, 25200072) to first detach cells from tissue culture dish. Adherent cells (BOSC 23) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, #11960) supplemented as described above for RPMI. For long-term storage, 5x10^6 cells were frozen in liquid nitrogen in 1ml of FBS containing 10% dimethyl sulfoxide (DMSO, MP Biomedicals, Solon, Ohio, #191418).
2.3.2 DNA transduction and enrichment

J558µm3 or Wehi231 cells were transduced with mutated or WT Cx43-EGFP plasmid DNA (Table 2.1) using the retroviral packaging cell line, BOSC 23. DNA was introduced into BOSC 23 cells by calcium phosphate precipitation as has previously been described (Krebs et al., 1999). Viral supernatant was collected at 24, 48, and 72 hrs post-transfection, filtered through a 0.2 µm syringe filter (VWR, Edmonton, Alberta, #28145-501) and used to infect 0.5x10^6 J558µm3 or Wehi231 cells (Table 2.2). Transduced B cells were sorted by fluorescence-activated cell sorting (FACS) by selection of EGFP positive (+) cells. Cell populations were periodically re-sorted to maintain high levels of expression (see Section 2.5.1).

J558µm3 cells were transfected with mutant Rap constructs using Nucleofection ®. Cells were re-suspended in the appropriate solutions from the Amaza Nucleofection Kit T (Amaza Biosystems, Gaithersburg, Maryland, #VCA1002) containing 2µg DNA according to the manufacturer's instructions. The parameters from the G-16 program in the Amaza Nucleofector Device (Amaza Biosystems) were used.
Table 2.2 Transduced cell lines used in study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expressing</th>
<th>Retroviral vector</th>
<th>Promoter</th>
<th>Bacterial resistance</th>
<th>Eukaryotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>J558µm3</td>
<td>-</td>
<td>AP2</td>
<td>CMV</td>
<td>AmpR</td>
<td>-</td>
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<tr>
<td>Cx43-EGFP</td>
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<td>Cx43T154A-EGFP</td>
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<td>Cx43Y265D-EGFP</td>
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<td>Wehi231</td>
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<tr>
<td>Cx43-EGFP</td>
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<td>Cx43T154A-EGFP</td>
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2.3.3 Cell stimulation

For stimulation of cells by cross-linking the BCR, cells were washed once and suspended in 0.5 ml quinsaline (25 mM sodium Hepes pH 7.4 (Sigma, #H3375), 125 mM NaCl, 5 mM KCl (Fisher, #P217), 1 mM CaCl₂ (Sigma, #C1016), 1 mM Na₂HPO₄ (Fisher, #S374), 0.5 mM MgSO₄ (Fisher, #M63), 1 g/L glucose, 2 mM L-glutamine (Sigma, #C8540), 1 mM sodium pyruvate, 50 µM β-mercaptoethanol). For each time-point in the given time-course, 5x10⁶ cells were aliquoted into a 1.7 ml microcentrifuge tube (Axygen Inc, Union City, California, #MCT-175-C) and were stimulated by crosslinking the cell surface BCR with 20 µg/ml goat α-mouse IgM and incubating the cells in a 37°C water-bath. Reactions were stopped by addition of 0.5 ml of
1:100 sodium pervanadate (Sigma, #13721-39-6) and cells were lysed for biochemical detection (Section 2.4) of changes downstream of stimulation through the BCR.

### 2.3.4 Drug treatment to block hemichannel function

J558μm3, Wehi231, A20, or primary murine B cells were incubated 15-30 min at 37°C with 5% CO₂ in RPMI medium containing: 100 μM HC-blocking drug carbenoxolone (CBX)(carbenoxolone disodium salt, Sigma, #C4790), 1 mM Panx HC-blocking drug probenecid (Pbn)(Alfa Aesar, Ward Hill, Massachusetts, #B20010), or 200 μM Cx HC-blocking-ion lanthanum (La³⁺)(LaCl₃, Sigma, #211605). Stimulated cells were either added directly to anti-BCR coated coverslips for spreading assay (see Section 2.5.1), or washed once in 1x phosphate buffered saline (PBS)(Gibco, Invitrogen, #10010-023) for assay of HC activity (see Section 2.6.3).

### 2.4 Biochemical procedures

#### 2.4.1 Cell lysis and preparation of samples

For western blotting, cell lysis was performed as has previously been described (Machtaler et al., 2011). Briefly, 5X10⁶ cells were lysed in 200-300 μl cold lysis buffer (1x PBS, 1% Triton-X 100 (Fisher, #BP15), 1% IGEPAL (Sigma, #CA-630), 50 mM CaCl₂ (Troxell et al 1999) containing protease inhibitors (10 μg/ml leupeptin (Sigma, #L2884), 1 μg/ml aprotinin (Roche, Mississauga, Ontario, #981532), 1 mM pepstatin A (Sigma, #P4265), 1 mM sodium
vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF)(Roche, #837091)). Samples were sonicated 10 sec with a Misonix XL sonicator ultrasonic cell processor (Misonix Incorporated, Farmingdale, New York) and incubated on ice for 10 min. To remove cellular debris, cell lysates were centrifuged 10 min at 4°C 15,000 g and pellets were discarded. To determine protein concentration, 10 µl of lysate was measured using a bicinchoninic acid (BCA) kit according to the manufacturers instructions (Pierce Biotechnologies, Rockford, Illinois, #23225). Five-times concentrated reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 4% glycerol (Fisher, #BP229-1), 2.5% sodium dodecyl sulfate (SDS)(Bio-Rad, #161-0301), 0.02% bromophenol blue, 100 mM diithiothreitol (Sigma, #D0632)) was added 1:5 to samples and were incubated 1 h in a 37°C water bath. Samples were immediately analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see Section 2.4.2).

2.4.2 SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting

Incubation with reducing sample buffer was used to denature proteins and coat them with a net negative charge. From each sample, 30 µg of protein was loaded as determined by the BCA assay, (see Section 2.3.1) and separated at 200 V for approximately 1 h, through an 8-12% SDS-polyacrylamide gel using a dual vertical min-gel apparatus with at water-cooling system (CBS Scientific, Del Mar, California), in running buffer (50 mM Tris, 0.4% glycine, 0.1% SDS). Precision Plus Protein Kaleidoscope standard (Bio-Rad, #161-0375) or BlueEye Prestained Protein Ladder (FroggaBio, GeneDireX, Toronto, Ontario, #PM007-0500) was loaded beside the samples and used to estimate molecular weight. Separated proteins were transferred to nitrocellulose membrane (Bio-Rad, #162-0115) using a Bio-Rad Mini Trans-
Blot® transfer apparatus and running a current of 20 V overnight in transfer buffer (20 mM Tris-HCl, 50 mM glycine, 20% methanol (Fisher, #A412-4)).

After transfer was complete, the nitrocellulose membrane was blocked 30 min in Tris-buffered saline (TBS)(2.5 g/L Tris, 8.8 g/L NaCl, pH 8) containing 5% skim milk powder or 5% bovine serum albumin (BSA)(Fisher, #BP1600) at room temperature, on a Lab Line Orbit Shaker (Lab Line Instruments Inc., Melrose Park, Illinois). Membranes were incubated overnight at 4°C in TBS containing 5% milk or 5% BSA, and primary antibody (Table 2.2), after which, excess primary antibody was removed by three successive 10 min washes in TBS containing 0.1 % Tween (Fisher, #BP337). Horseradish peroxidase (HRP)-conjugated secondary antibody (Table 2.2) was added in TBS containing 5% milk for 1 h at room temperature, with shaking. Excess secondary antibody was removed by another three 10 min washes in TBST. Nitrocellulose membranes were incubated for 1 min in a minimum volume of 1 ml Amersham ECL Western Blotting Detection Reagent (GE Healthcare, #RPN2106VV1/2), and were then exposed for various time periods in a darkroom using 8 x 11” Classic Blue Autoradiography Film BX (Mandel Scientific, Guelph, Ontario, #EBA45) and developed with a Kodak X-OMAT 1000A processor (MedTec Marketing Group, Burnaby, British Columbia). Re-probing was performed after stripping membranes by three 20 min washes in TBS pH 2.
Table 2.3 List of antibodies used in western blotting.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Conjugation</th>
<th>Concentration</th>
<th>Host</th>
<th>Company</th>
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<td><strong>Primary Antibodies:</strong></td>
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<td></td>
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<tr>
<td>Cx43 C-terminus</td>
<td>-</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Sigma (C6219)</td>
</tr>
<tr>
<td>Cx43 N-terminus</td>
<td>-</td>
<td>1:250</td>
<td>Mouse</td>
<td>Fred Hutchinson (Seattle Washington)</td>
</tr>
<tr>
<td>Cx43 pY265</td>
<td>-</td>
<td>1:1000</td>
<td>Rabbit monoclonal</td>
<td>(Solan and Lampe, 2008)</td>
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<tr>
<td>IgM (µ chain)</td>
<td>-</td>
<td>1:3000</td>
<td>Goat</td>
<td>Jackson Immuno Research (115-005-020)</td>
</tr>
<tr>
<td>Akt</td>
<td>-</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Cell Signaling (9272)</td>
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<tr>
<td>Actin</td>
<td>-</td>
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<td>Mouse</td>
<td>Fisher (ICN691001)</td>
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<tr>
<td>pY (4G10)</td>
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<td>1:2000</td>
<td>Mouse monoclonal</td>
<td>In-house (Morrison et al., 1989)</td>
</tr>
<tr>
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<td>-</td>
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<td>Rabbit</td>
<td>Cell Signaling (2399S)</td>
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<tr>
<td><strong>Secondary Antibodies:</strong></td>
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<tr>
<td>Mouse IgG</td>
<td>HRP</td>
<td>1:3000</td>
<td>Goat</td>
<td>Bio-Rad (170-6516)</td>
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<tr>
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<td>HRP</td>
<td>1:3000</td>
<td>Goat</td>
<td>Bio-Rad (170-6515)</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>HRP</td>
<td>1:3000</td>
<td>Donkey</td>
<td>Santa Cruz (SC-2020)</td>
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</table>

2.4.3 Phosphatase treatment

J558µm3 cells expressing WT or mutated Cx43-EGFP were stimulated for various lengths of time with goat α-mouse IgM and lysed as described previously (see Sections 2.3.3 and 2.4.1 respectively). For each time-point, 30 µg of protein was aliquoted each, one for phosphatase-treatment and one for an untreated control. Thirty units of calf-intestinal phosphatase (CIP, New England BioLabs, Ipswich, Massachusetts, #M02905) were added to phosphatase-treated samples and NEB buffer was added to all samples, followed by 1 h incubation in a 37°C water bath. Reducing sample buffer was added after phosphatase treatment and
samples were incubated an additional 1 h at 37°C. Samples were analyzed immediately by SDS-PAGE and western blotting (Section 2.4.2).

2.4.4 Biotinylation of surface proteins

To detect cell surface proteins, biotinylation was performed as has been described previously (Condon et al., 2000). For each sample, 2.5-5x10⁷ cells were washed twice with 1x PBS and re-suspended in either 3 ml of 1x PBS containing 0.5 mg/ml sulfo-NHS-biotin (Pierce, #21217) or 1x PBS alone in 15 ml polypropylene tubes (BD Falcon, #430791) and rocked for 1 h at room temperature. Unbound biotin was removed by three successive washes with 3 ml of 1x PBS containing 2 mg/ml lysine. Cells were lysed as described above (see Section 2.4.1) and 40 µl of lysate was set aside as a control. The remaining lysate was pre-cleared of non-specific binding by 1 h incubation with un-conjugated beads. Beads were prepared by pelleting 30 µl of agarose beads (Vector Laboratories, Burlingame, California, #94010) by centrifugation 1 min at 14,000 g in 1.7 ml micro-centrifuge tubes and by two successive washes with 300 µl 1x PBS. Lysates were added to the washed beads and rocked for 30 min at room temperature. Following centrifugation to remove beads, cleared supernatant was collected and added to 30 µl washed avidin-conjugated agarose beads (Peirce, #20219). Avidin-beads were rocked for 1 h at room temperature with pre-cleared lysates to bind biotin, after which beads were washed three times in 300 µl lysis buffer, dried completely, and suspended in 30 µl of 1x reducing sample buffer to remove protein from beads and to prepare them for SDS-PAGE and western blotting. Samples were either boiled for 5 min or
incubated in a 37°C water bath for 1 h depending on whether Cx43 or other surface proteins were being detected respectively. Samples were analyzed by western blot (Section 2.4.2).

### 2.4.5 Immunoprecipitation

Cells were stimulated and lysed as previously described (Sections 2.3.3 and 2.4.1 respectively). To prevent non-specific binding, lysates were pre-cleared by 1 h of rocking at 4°C with 30 µl of protein A-conjugated sepharose beads (Sigma, #P9424). Beads were discarded after pelleting by centrifugation at 14,000 g for 2 min. For IP, 3 µg of rabbit α-mouse Cx43 (Sigma, #C6219) was added to cleared lysates and rocked gently at 4°C for 1 h in a total volume of 1 ml to ensure sufficient mixing. Another 30 µl of protein A-conjugated sepharose beads were washed once in 200 µl lysis buffer and added to lysates to bind the antibody-bound Cx43 protein. Bead/antibody/lysate mixture was rocked at 4°C overnight and then centrifuged for 2 min at 14,000 g to collect beads. Beads were washed three times in 200 µl lysis buffer, dried well, and re-suspended in 30 µl of 1x reducing sample buffer. Samples were incubated 1 h in a 37°C water bath to detach proteins from antibodies and beads and to prepare protein for SDS-PAGE and western blotting (Section 2.4.2).

### 2.4.6 Rap activation assay

Rap activation was performed as has been described previously (McLeod et al., 1998). Briefly, 0.5x10^6 cells/time-point were stimulated (Section 2.3.3) and lysed in 500 µl radioimmunoprecipitation assay (RIPA) lysis buffer (30 mM Tris-HCl pH 7.4, 150 mM NaCl,
1% IGEPAL, 0.5% deoxycholate (Sigma, #D6750), 0.1% SDS, 2 mM EDTA) containing protease inhibitors (10 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM pepstatin A, 1 mM Na$_3$VO$_4$, 1 mM PMSF). The Rap1 binding domain of the Ral-GDS protein was used to pull down only the active, GTP-bound form of Rap. Ral-GDS was purified using the pGEX-RalGDS (RBD) plasmid as has been described previously (McLeod et al., 1998). Ral-GDS beads were made fresh by adding 20 µl of Ral-GDS and 30 µl of washed glutathione-sepharose 4B beads (GE Healthcare, #17-C756-01). 30 µl lysate was set aside for measuring total Rap and remaining lysates were added to beads for pull-down. After boiling in reducing sample buffer, samples were loaded immediately or frozen at -20°C. Samples were analyzed by western blot (Section 2.4.2) and probed with an antibody against total Rap (Table 2.3).

2.5 Flow cytometry

2.5.1 Sample preparation and staining

For surface staining, 1x10$^6$ J558µm3 cells expressing WT, mutant Cx43-EGFP or empty vector were washed 1x in PBS by centrifugation at 1500 rpm for 5 min, and re-suspended in 50 µl of FACS buffer (1x PBS containing 2% FBS) in a 96-well round-bottomed plate (BD Biosciences, #353917). PE-conjugated α-IgM (1.25 µg/ml) was added and cells were incubated for 30 min on ice, protected from light. To wash cells, 100 µl of FACS buffer was added for a final volume of 150 µl and the plate was spun at 1,500 rpm for 5 min in a H-103N series silencer plate spinner (Western Scientific, Valencia, California). The plate was inverted to discard supernatant and pelleted cells were washed an additional two times in 150 µl FACS buffer.
before re-suspending in 300 µl FACS buffer in polystyrene or polypropylene FACS tubes (BD Falcon, #352054 or #352063 respectively). Stained cells were transported on ice, protected from light to the UBC Flow Cytometry Facility (www.ubcflow.ca)

2.5.2 Data collection and analysis

Flow cytometry was performed regularly to check cell lines (Table 2.2) for expression of transduced constructs (WT or mutant Cx43-EGFP and IgM). Igα expression was found to be stable and did not need re-sorting, and Igβ is endogenously expressed by J558 cell lines. Cells were analyzed on a BD LSRII Flow Cytometer (BD Biosciences) using BD FACS Diva software (BD Biosciences). Expression of Cx43-EGFP and IgM were determined by measuring the percentage of the population EGFP+ and PE+ respectively. Gating was used to exclude debris and dead cells based on forward scatter/side scatter plot generated using FlowJo Flow Cytometry Analysis Software (Tree Star Inc., Ashland, Oregon). Cells were re-sorted by the UBC FLOW FACS facility to prevent periodic loss of either Cx43-EGFP or IgM expression.

2.6 In vitro B-Cell assays

2.6.1 BCR-mediated cell spreading

To prepare the spreading assay, the appropriate number of 12 mm glass coverslips (Fisher, #12-545-80) were sterilized in 100% methanol in a 24-well polystyrene tissue culture dish (BD Falcon, #353047), dried completely, and coated overnight shaking in 500 µl of 1x PBS
with 40 µg/ml of goat α-mouse IgM. Coverslips were washed three times in 1x PBS and 2-3x10^5 cells were added in 1 ml RMPI and incubated at 37°C with 5% CO₂ for the given time-point. Cells were fixed in 250 µl 4% paraformaldehyde (Cedar Lane Labs, Burlington, North Carolina, #15710) for 10 min, washed once in 1x PBS and then permeabilized in 0.5% Triton-X 100 for 10 min. Cells were washed once in 1x PBS and then a 30 min incubation in 200 µl of 1:40 rhodamine-phalloidin (Molecular Probes, Invitrogen, #R415) was used to stain actin. Coverslips were washed a final three times and mounted onto 3 x 1” glass microscope slides (Fisher, #12-550-123) using ProLong Gold anti-fade reagent supplemented with DAPI (Molecular Probes, Invitrogen, #P36935) to stain the nucleus. Samples were dried overnight, immobilized with nail polish, and slides were stored at -20°C. The contact area between the adhered or spreading cells and the coverslip was imaged using an Olympus Fluoview 1000 confocal microscope viewed using the 60x objective. Contact area was quantified using Image Pro Plus 6.2 analysis software (Media Cybernetics, Rockville, Maryland) and expressed as µm².
Figure 2.1 Schematic of B-cell spreading assay. J558µm3 cells do not express Cx43 and were used as a gain of function model to study the ability of WT versus mutant Cx43 to allow B cell spreading on glass coverslips coated with α-IgM (the BCR type expressed by J558µm3). The contact site between cells and coated coverslips was imaged with a confocal microscope and contact areas were measured via actin (rhodamine-phalloidin) staining.

2.6.2 Cell proliferation

An aliquot per day of 1×10³ cells in 50 µl of RPMI were incubated for four days in a 384-well plate (BD Falcon, #353962) at 37°C with 5% CO₂. To stain live cells, 5 µl of 0.01% alamar blue (resazurin sodium salt, Sigma, #R7017) was added each day, and colour change was measured at A₅₉₀ after a four-hour incubation using a SPECTRAmax Gemini-XS spectrophotometer (Molecular Devices, Sunnydale, California). Live cells were measured over a 4-day period.
2.6.3 Hemichannel assay

Channel activity was measured using the procedure described by (Contreras et al., 2002) and adapted by Dr. Jose Luis Vega Pizarro (Universidad de Catolica de Chile, Santiago Chile, visiting Postdoctoral Fellow with Dr. Christian Naus lab, Department of Cellular and Physiological Sciences, UBC) and myself. For measuring dye-uptake, 5 million cells were centrifuged at 1500 rpm 5 min and washed once in 1x PBS. Cells were re-suspended in 2 ml of Locke’s buffer (9 g/L NaCl, 0.4 g/L KCl, 0.2 g/L NaHCO₃) with or without divalent cations (0.25 g/L CaCl₂) in a poly-D-lysine coated 35 mm glass bottom microwell dish (MatTek, Ashland Massachusetts, #P35GC-1-14-C) and focused on with a Zeiss Axioplan 2 Imaging microscope (MicrOptik, Deursen Netherlands) (16x objective). To begin HC assay, 1 ml of Locke’s buffer containing 3x concentrated (15 µM) ethidium bromide (Invitrogen, #15585011) was added for a final concentration of 5 µM and images were collected every 30 sec for 15 min using Image J software.

2.6.4 Cell aggregation

To coordinate cell density between cell types, 5x10⁵ cells/ml were grown overnight in 10 cm tissue culture dishes. To measure aggregation, 2.5x10⁵ cells from each dish were transferred to one well of a 6-well tissue culture dish containing 2 ml of RMPI media containing reduced serum (1% FBS) to prevent cell division. Cells were dispersed by pipetting up and down, and were rocked at 150 rpm at 37°C. Images were taken at 0, 5, 24, and 48 h. Cell aggregation was measured by quantifying the area of each object (aggregate of cells), dividing by the diameter.
of a single cell, in order to calculate the number of cells/clump (Figure 2.2). Quantification was performed using Image Pro 6.2.

![Figure 2.2 Schematic of quantification of cell aggregation.](image)

Each clump of cells was counted as an object. Object areas were divided by the smallest object area measured at time zero, which corresponded to the area of a single cell, giving the number of cells/object. A sample calculation is given.
2.6.5 Bead clearing cell motility assay

18 mm glass coverslips (VWR, #48380-046) were coated overnight at 4°C with 20 µg/ml (2.5 µg/cm²) bovine fibronectin (Invitrogen, #F1141) in 1X PBS. The coverslips were washed once with 1X PBS and coated with 6 µl of fluorescent blue FluoSpheres (Molecular Probes, Invitrogen, #F8815) suspended in 100 µl 1X PBS per coverslip. Complete media containing either 100 ng/ml SDF-1 or no cytokine and 1500 cells were added to the coated coverslip and incubated for 18 h at 37°C in 5% CO₂. Cells were fixed and stained as described for the cell spreading assay (Section 2.6.1). Images were obtained using an Olympus BX61 microscope (Olympus) with the 20X objective, and the area of the beads that were cleared were quantified using cellSens Dimension image analysis software (Olympus).

2.7 Confocal microscopy

2.7.1 Sample preparation and staining

For intracellular staining, cells were immobilized on 12 mm glass coverslips that had been sterilized with 100% methanol and coated for 30 min with 500 µl of 1:10 poly-L-lysine (Sigma, #P4707). Coverslips were washed three times in 1x PBS and 3x10⁵ cells were added and allowed to settle and adhere to the coated-glass for 5 min. Cells were fixed in 250 µl of 4% paraformaldehyde in 1x PBS for 10 min. Excess paraformaldehyde was removed by washing coverslips once in 1x PBS. Cells were permeabilized for intracellular staining in 0.5% Triton-X 100 for 10 min and washed once in 1x PBS. For staining of the BCR, cells were
blocked by 30 min incubation in 1x PBS containing 3% BSA. Primary antibody (200 µl of 1:200 goat α-mouse IgM) was added for 30 min in 1x PBS+3% BSA, was washed away three times, followed by a 30 min incubation with 200 µl of 1:200 Alexa647 rabbit α-goat IgG (Molecular Probes, Invitrogen, #A21446).

For staining of the endoplasmic reticulum (ER), 3x10^5 cells were centrifuged 5 min at 1,500 rpm and re-suspended in 500 µl of 1x PBS containing 0.5 µl ER Tracker (Molecular Probes, Invitrogen, #E12353) in a 24-well polystyrene tissue culture plate. Cells were incubated 30 min at 37°C at 5% CO₂ for labeling of the ER, 5 min in 1x PBS to wash away excess stain, and a final 5 min in 500 µl RPMI, and then added to poly-L-lysine-coated coverslips for 5 min. Poly-L-lysine-coated coverslips were prepared as described above. Cells were fixed as described above and mounted onto glass slides in anti-fade reagent. Slides were stored at -20°C.

2.7.2 Image acquisition and analysis

For confocal microscopy, cells and coverslip were imaged using an Olympus Fluoview 1000 confocal microscope with the 60x objective. The contact area was quantified using Image Pro 6.2 analysis software (Media Cybernetics, Rockville, Maryland). Microscope and imaging software were part of the Life Sciences Institute Imaging Facility (http://facilities.lsi.ubc.ca/core-facilities/imaging).
HC assays were performed in collaboration with Dr. Jose Luis Vega Pizarro and the Naus lab. Live cell imaging was performed on a Zeiss Axioplan 2 microscope with the 16x objective. Dye-uptake was quantified using Image J analysis software.

2.8 Statistics

To analyze significance, a student’s two-tailed, unpaired t-test was used to compare the means to either a positive or negative control using Microsoft Excel. Asterixes (*) represent significance based on a 95% confidence interval (P<0.05). Error bars on graphs show standard error of the mean.
CHAPTER 3
RESULTS

This thesis project is based on previous results in the lab that demonstrated the importance of Cx43 in B-cell processes that depend on cytoskeletal rearrangement such as cellular adhesion, spreading, and migration. It is unclear whether Cx43 influences these processes by acting 1) as an adhesive protein using its extracellular domains, 2) as a constituent of functional GJs/HCs, or 3) as a scaffold to promote protein-protein interactions through its CT tail. To differentiate between these possible explanations, regions of the Cx43 amino acid sequence were targeted by mutating 1) the extracellular loops, 2) the transmembrane domain that lines the GJ or HC pore, and 3) the cytoplasmic CT tail.

The first target: the extracellular loops were investigated by making mutations of conserved cysteine residues that are thought to be involved in cell adhesion events including those in forming GJ pores. These mutations, when expressed in B-lymphocytes, resulted in intracellular accumulation of Cx43 probably due to defective trafficking of Cx43 proteins harboring these mutations through the secretory pathway. Due to the severity of these trafficking defects and the importance of surface expression for our assays, that rely on interactions with the BCR on the plasma membrane, it was decided to abandon further work on this panel of mutants, and instead, to focus on the channel-lining (Chapter 3.1) and cytoplasmic CT (Chapter 3.2) domains of Cx43 which when mutated, both appeared to traffic normally to the cell surface. The work performed making the extracellular domain mutants and their characterization are summarized in Appendix 1.1.
3.1 BCR-mediated B-cell spreading is independent of Cx43 channel function.

The second target was the channel domain, and was investigated using the Cx43 closed-channel mutant Cx43T154A. Our results suggest that this mutation may influence BCR-mediated cell spreading through a yet undefined conformational defect to the CT tail.

3.1.1 Rationale

Connexins (Cxs) have traditionally been considered building blocks of GJ channels, whose purpose is to facilitate cell-cell communication (Evans and Martin, 2002). Although mature B-cells only make relatively transient GJ contacts such as those formed during transmigration through endothelium (Oviedo-Orta et al., 2002) and during immune synapse formation with antigen presenting cells like follicular dendritic cells (Krenacs et al., 2005) or T-cells (Oviedo-orta et al., 2008), a number of reports show that Cxs may form HCs that connect the cell with the extracellular environment. These studies, however, are often criticized due to the difficulty in measuring GJ communication and the difficulty in linkage to relevant physiological processes (Spray et al., 2006).

We have previously found that the Cx43 CT tail is important for B-cell adhesion, spreading, and motility (Bruzzone et al., 2003; Machtaler et al., 2011). Truncation of the CT domain of Cx43 at aa 246 (Δ246) leaves the microtubule-binding domain intact and does not appear to alter trafficking of the mutated Cx43 to the plasma membrane nor its ability to form GJs (Bates et al., 2007), however truncation of the CT domain has been shown to reduce GJ
conductance in some cases (Behrens et al., 2010; Crespin et al., 2010). We propose that the Cx43 CT tail may play a role in gating HC activity or selectivity and that point mutations of the CT domain may alter the ability of the Cx43 CT domain to influence either opening or closing of HCs, either by its contribution to the structure of Cx43, or by influencing the binding of proteins to the CT tail which then can regulate HC activity.

To address the importance of the channel in BCR-mediated B-cell spreading, we used two approaches: First a pharmacological approach was utilized, and B-cells were treated during BCR-mediated spreading with drugs that are known to block channel activity. These drugs allow us to impede the function of endogenous or overexpressed HCs in various B-cell types, but lack specificity and can block other types of HCs, therefore we also used a genetic approach by expressing a Cx43 mutant that was unable to form functional channels in both a Cx43-null B-cell line (plasmacytoma cell line: J558µm3, expressing a mIgM BCR) and a Cx43 expressing B-cell line (B-lymphoma cell line: Wehi231).

### 3.1.2 Channel-blocking drugs did not prevent B-cell spreading

To test the importance of HC function of Cx43 in B-cell spreading, three channel blocking agents were used. The ion lanthanum (La3+) blocks Cx HCs whereas the drug probenecid (Pbn) blocks Panx HCs, and the drug carbenoxolone (CBX) blocks both types of HCs. Wehi231 cells, A20 cells, and primary splenic B-cells isolated from C57BL/6 mice were pre-
Figure 3.1 BCR-mediated B cell spreading is not affected by hemichannel-blocking drugs. BCR-mediated cell spreading on α-IgM (BCR) coated glass coverslips for 0 or 30 min by A) A20 cells B) Wehi231 cells and C) Primary splenic B cells isolated from C57BL/6 mice D) J558µm3 cells expressing Cx43-EGFP (J558µm3+Cx43-EGFP cell spreading shown in E). Contact area between cells and the coverslip were quantified from images of rhodamine-phalloidin stained actin (red) to visualize the spreading cell periphery taken using an Olympus Fluoview 1000 confocal microscope using a 60x objective. (Error bars represent standard error of the mean). Data is representative of three (A-C) or one (D and E) independent replicates where >100 cells were quantified per sample. Asterix denotes significance between treatments as determined by P-value <0.05 by a Student’s two-tailed, unpaired t-test calculated by Excel (no significant differences).
treated with these blocking agents to induce closure of HCs prior to incubation on anti-IgM (anti-BCR) coated glass coverslips (for detailed methods, see Chapter 2.3.4). For all B-cell types, cells treated with HC-blockers spread as well as untreated cells (Figure 3.1) and remained viable based on the absence of nuclear fragmentation detected by staining with DAPI, which would indicate apoptosis (Data not shown).

Because the majority of studies of HC activity have been performed in adherent cell types, we collaborated with an expert in HC research, Dr. Jose Luis Vega Pizzaro (Universidad de Catolica de Chile, Santiago Chile, and a visiting Postdoctoral Fellow in Dr. Christian Naus’s lab, Department of Cellular and Physiological Sciences, UBC) to adapt an assay of HC activity used for adherent cells for use in B-lymphocytes.

3.1.3 Overexpressed Cx43 forms hemichannels that are not actively regulated by removal of divalent cations or BCR-mediated spreading in B-cells

Ethidium bromide (EtBr) uptake was used to measure HC activity in B-cells (for method see Chapter 2.6.3). EtBr was used over other fluorophores because it is a nucleic acid intercalating agent and fluoresces only after binding to DNA. This allows us to measure dye-uptake into the nucleus as a measure of HC internalization, without washing cells since the dye will only fluoresce in cells where it binds DNA, and not when in solution.
Figure 3.2 Hemichannel activity in B-cell lines. Rate of dye uptake was calculated by taking the slope of fluorescence over time for each set of conditions (basal uptake in white, activation by divalent cation-free solution (DCFS) in black, treatment with HC-blocking drug carbenoxolone (CBX) in grey, and spreading on anti-IgM (BCR) in checkered). Error bars represent standard error of the mean. Data is represents one to four replicates per condition. (Data by Dr. Jose Luis Vega Pizarro, visiting Postdoctoral Fellow, Universidad de Catolica de Chile, Santiago Chile, with Dr. Christian Naus lab, Department of Cellular and Physiological Sciences, UBC).
This modification of the assay was essential for working with cells in suspension since it allowed us to measure the same cells before and after EtBr addition, whereas washing would have required centrifugation and re-suspension of the cells, making it impossible to compare the same cells before and after addition of the dye (EtBr).

The mean of the slope of dye uptake/time was plotted to show the rate of dye-uptake (Figure 3.2). Primary C57BL/6 murine astrocytes, which are adherent cells, were used as a positive control of HC activity (for review, see (Bennett et al., 2003)) in our assay, and compared with various B-cell lines. A20 cells (another B-lymphoma cell line) and J558µm3+EGFP (vector control cells only transfected with the EGFP tag) cells had similar rates of dye uptake as astrocytes in basal conditions (no uptake), and were not activated by removal of divalent cations (divalent cation free solution: DCFS (no Ca²⁺Mg²⁺)). In contrast, J558µm3+Cx43-EGFP cells had high levels of dye-uptake in basal conditions but this uptake was not further activated by DCFS. Dye-uptake by J558µm3+Cx43-EGFP was blocked by pre-treatment with 100 µM CBX, a HC blocker, showing that dye-uptake occurs specifically through HCs in these cells. Comparison with J558µm3+EGFP confirmed that dye uptake was specific to Cx43 HCs.

Overexpression of Cx43-EGFP seemed to cause formation of Cx43 HCs, yet dye-uptake is not enhanced by the removal of divalent cations, which is a common HC activator used in astrocytes and other cell types (Verselis and Srinivas, 2008; Ye et al., 2003). One possibility explaining this lack of response is that the overexpression of Cx43 caused a “maxed-out” HC response that could not be enhanced by further activation, another could be that removal of divalent cations does not activate HCs in B-cells. While removal of divalent cations is one of
the most widely used activators of HCs, other conditions have been shown to regulate HC activity in specific cell types. Fluid flow shear stress has been shown to activate HCs in fibroblasts through integrin signaling, for example (Batra et al., 2012).

Since cell spreading creates tension on the plasma membrane, we asked whether the force generated during B-cell spreading might be sufficient to cause activation of Cx43 HCs. J558µm3+Cx43-EGFP cells spreading on α-IgM coated glass dishes did not take up more dye than cells in basal conditions (Figure 3.2), indicating that HC activity in J558µm3+Cx43-EGFP cells is not dependent on cell spreading or BCR signaling, but is a result of Cx43 overexpression.

While J558µm3+Cx43-EGFP cells form HCs, this seems to be an overexpression phenotype since dye-uptake was not observed in HC assays with un-transduced J558µm3 cells or in the other B-cell lines A20 and Wehi231 (Wehi231 data not shown).

### 3.1.4 Expression of Cx43T154A in a B-cell line: J558µm3

To investigate the importance of channel activity to B cell spreading, we decided to use the well-studied Cx43 point mutation T154A. The Cx43T154A mutation was characterized by Dr. Gina E. Sosinsky’s lab (Department of Neurosciences, University of California, San Diego; (Beahm et al., 2006)) as a point-mutation within the third (TM3) region of Cx43 and was identified by a larger mutagenesis study (Skerrett et al., 2002). This mutated Cx43 can still form GJ plaques but prevents dye-transfer in between *Xenopus* oocytes (Beahm et al., 2006).
Other mutations of this conserved threonine, for example Cx26T135A, shows structural anomalies in GJ plaque formation and stability of oligomers (Beahm et al., 2006).

With regard to the Cx43T154A mutation, preliminary experiments were performed in B-cells by previous MSc student Caren Jang, and I extended these studies. We both used an expression vector containing a cDNA encoding Cx43 with the T154A mutation (Chapter 2.1.1) from Dr. Gina E. Sosinsky’s lab (Beahm et al., 2006). In addition, site-directed mutagenesis was performed on WT Cx43 in order to generate the T154A mutation in Cx43-EGFP expressed in the NAP2 expression vector (Bates et al., 2007) for consistency with other Cx43 constructs that had the EGFP tag. To investigate the role of the channel mutant on B-cell spreading, this plasmid was expressed in J558µm3 cells by retroviral infection. Populations of cells were enriched for those expressing Cx43T154A-EGFP by FACS sorting for the EGFP+ cells, and Cx43 expression was confirmed by western blotting (Figure 3.3 B).

To ensure that the Cx43T154A mutation did not alter trafficking and localization of Cx43 to the plasma membrane in J558µm3 cells, confocal images were taken and WT or mutant Cx43 were compared (Figure 3.3 C). WT and mutant Cx43 were visualized via their EGFP tag, and co-localization with both a live ER tracker and immunostained IgM (part of the BCR on the cell surface of J558µm3 cells). These immunofluorescence studies showed that the Cx43T154A mutation has no major deleterious effects on Cx43 trafficking from the ER to the plasma membrane, or co-localization at the plasma membrane with the BCR (containing IgM
Figure 3.3 Characterization of J558μm3 cells expressing Cx43T154A. A) Schematic showing point mutation of threonine 154 to alanine. B) Western blot showing expression of Cx43T154A in the J558μm3 cell line populations stably transduced by retroviral infection (Blot was stripped and re-probed for actin as a loading control). C) Localization of EGFP-fused WT Cx43 or Cx43T154A expressed by J558μm3 cells viewed by an Olympus Fluoview 1000 confocal microscope using a 60x objective. Green=WT or mutant Cx43-EGFP, red=ER (middle panel) or IgM (BCR) (bottom panel), yellow=merge, blue=DAPI stained nuclei. Scale bars represent 10 μm. Data shown is representative of three independent replicates.
heavy chain). This was further confirmed using biochemical methods (see Section 3.1.5 below).

To ensure that Cx43T154A mutation didn’t alter cell size or proliferation, J558µm3 cells expressing Cx43T154A, WTCx43, and EGFP alone were compared (Figure 3.4). Loss of Cx43 has been associated with increased tumorigenicity (Rose et al., 1993), whereas overexpression of Cx43 in C6 rat glioma cell line caused a significant reduction in proliferation (Zhu et al., 1991). Proliferation using a live-cell dye, alamar blue uptake, was measured by a fluorescent plate reader (for detailed methods see Chapter 2.6.2), and we found that J558µm3 cells expressing Cx43T154A proliferated at the same rate over a 4-day period as cells expressing WT Cx43 or EGFP alone (Figure 3.4 A).

Since Cx43 was overexpressed in the J558µm3 B-cell line, we wanted to ensure that excess Cx43 at the plasma membrane didn’t cause an increase in cell size, which could confound the results of the spreading assay by influencing the cell area. Relative cell size was compared by flow cytometry (Chapter 2.5) using the forward scatter measurement (FSC). WTCx43 and Cx43T154A expression, when compared to EGFP expression by J558µm3 cells, did not affect cell size when cells were not stimulated by the BCR (Figure 3.4 B), thus confirming that any differences that may arise between EGFP, Cx43, and Cx43T154A during the cell spreading assay, can be attributed to the BCR-stimulated spreading response itself and not an unexpected affect on overall cell size.
**Figure 3.4 Expression of Cx43T154A by J558μm3 does not affect cell size or proliferation.**

A) Cx43T154A expression did not affect proliferation over a 4-day period, measured by Alamar blue uptake and quantified using a fluorescent plate reader. Error bars represent standard error of the mean. (Data is representative of three separate experiments, each done in triplicate). B) Cell size, measured by forward scatter (FSC) using flow cytometry. Data is representative of three separate experiments, each measuring 20,000 cells. Asterix denotes significance as determined by P-value <0.05 by a Student’s two-tailed, unpaired t-test calculated by Excel (no significant differences).
3.1.5 Cx43-EGFP is expressed at the cell surface in transduced J558µm3

It was important to confirm proper trafficking of the overexpressed Cx43-EGFP to the cell surface of J558µm3. Fusion of EGFP to the Cx43 cytoplasmic terminus has previously been shown to not impair trafficking or gap junction formation in NRK, MDCK, HeLa, and N2A cell types (Jordan et al., 1999; Laird et al., 2001) but it was important to confirm that Cx43 was present at the cell surface in a B-cell system. Confocal microscopy of EGFP-tagged Cx43 showed expression at what appears to be the plasma membrane (Figure 3.5 A), as well as in an intracellular aggregate that has previously been shown to co-localize with the ER as well as with early endosomes (Machtaler et al., 2011; Silverman et al., 2008).

To confirm whether Cx43 was expressed at the cell surface, intact cells were biotinylated to label surface proteins. The biotinylated cell surface proteins were isolated using avidin-coated beads under conditions in which intracellular proteins could not be further biotinylated (for detailed methods see Chapter 2.4.4). Next SDS-PAGE and western blotting were used to separate the lysed samples and proteins of interest were detected with antibodies (Table 2.2). As a positive control for this method surface IgM was detected from biotinylated, but not buffer-treated cells (data not shown). Cx43 was detected in biotinylated
Figure 3.5 Surface expression of Cx43-EGFP in B-cells. Surface expression of A) Cx43-EGFP overexpressed by J558μm3 cells and B) α-IgM stained endogenous BCR shown using an Olympus Fluoview 1000 confocal microscope using a 60x objective. Green=Cx43-EGFP, blue=DAPI stained nuclei, red=IgM (BCR). Scale bar represents 10 μm. (Localization has also been published, Caren Jang MSc thesis UBC 2008, Machtaler et al., 2011). Confirmation that Cx43 can be biotinylated on the cell surface using sulfo-NHS-biotin and pulled down using avidin-coupled beads as a method for isolating plasma membrane-bound proteins. Biotinylated proteins were isolated using avidin beads and then protein was denatured and removed from beads, immunoblotted and probed for Cx43 or Akt as a negative control (cytosolic protein) in the following cell lines: C) Wehi231 cell line, which has endogenous Cx43, was used as a positive control. D) Overexpressed Cx43-EGFP stably introduced into J558μm3 cells by retroviral infection is also expressed at the cell surface. Data shown represents one or two experiments (C or D respectively).
Wehi231 which expresses endogenous Cx43, as well as from transfected J558µm3+Cx43-EGFP. This shows that Cx43-EGFP is trafficking to the cell surface in J558µm3 cells. The cytosolic protein Akt/protein kinase B was used as a negative control for biotinylation since it should not be accessible to the biotinylation reagent during the process of labeling and was not found in biotinylated or buffer-treated samples that were precipitated with avidin, but was detected in total cell lysates (Figure 3.5). Akt was also useful as a gel-loading control. The MW of Cx43-EGFP from J558µm3 cells was larger due to its EGFP tag (Figure 3.5 D).

3.1.6 Expression of Cx43T154A causes non-radial BCR-mediated spreading in a B-cell line: J558µm3

Expression of the Cx43 mutant T154A had no effect on the average contact area as compared to expression of WT Cx43 in J558µm3 cells spreading on anti-IgM coated glass coverslips (Figure 3.6 B). However, Cx43T154A expressing cells would often extend a single long actin and EGFP-containing projection rather than spreading radially (Figure 3.6 A, arrows). (This spreading phenotype was also observed in preliminary spreading experiments performed by previous MSc student Caren Jang and myself, by J558µm3 cells expressing an untagged Cx43T154A from Dr. Gina E. Sosinsky, data not shown). When quantified, a significantly higher percentage of Cx43T154A-expressing cells formed these single projections, which were counted if the projection’s length measured at minimum, half the diameter of the cell (Figure 3.6 C, Caren Jang found that 50-60% of J558µm3 expressing Cx43T154A formed polarized protrusions in four additional independent experiments, data not shown).
Figure 3.6 Spreading of J558µm3 cells expressing Cx43T154A. A) Confocal images taken of the contact between cells and anti-IgM coated coverslips. Green=WT or mutant T154A Cx43-EGFP, red=rhodamine phalloidin stained actin, yellow=merge. Scale bars represent 10 µm. B) Quantification of the contact area between the cells and the coverslip. C) Percentage of cells exhibiting a non-radial spreading phenotype (arrows shown in A). Protrusions were considered if they exceeded a minimum length of half the cell diameter. D) Non-radial spreading was also quantified by a roundness measurement (length/width) 1=round, >1=non-round. Error bars represent standard error of the mean. Data is representative of five independent replicates in which >100 cells were counted per sample. Asterix (*) denotes significant difference between cell types as compared to EGFP-expression alone as determined by P-value <0.05 by a student’s two-tailed, unpaired t-test calculated by Excel.
Cx43T154A-expressing J558µm3 cells were on average, significantly less round than Cx43- or EGFP-expressing cells as determined by length/width ratio (Figure 3.6 D). Since this assay was performed on glass coated coverslips, as opposed to on a monolayer of cells with which the J558µm3 cells could form GJs with, this suggests that this B-cell spreading phenotype depends upon either a molecule transported through HCs (as opposed to GJs), or is due to a region of Cx43 that may be altered structurally by the Cx43T154A mutation.

3.1.7 Cx43T154A had a dominant negative effect on BCR-mediated B-cell spreading in a B-cell line: Wehi231

Evidence from the literature suggests that T154A mutated Cx43 can have dominant-negative (DN) affects by forming heteromeric channels with WT Cxs and interfering with normal functions. Cx37T154A blocks GJ communication by forming heteromeric channels with Cx43 and presumably with WTCx37 (Good et al., 2011). Interestingly, Cx37T154A also causes cell cycle arrest in a DN fashion; a process that depends on Cx37 binding to cell cycle regulatory machinery, not GJ communication (Good et al., 2011). The authors hypothesize that the Cx43T154A mutation changes the configuration of Cx37 such that interaction with adaptor proteins is no longer possible, and suggesting that Cx43T154A may have affects not exclusively due to its channel-blocking properties.

In order to determine if Cx43T154A can act as a DN when interacting with WT Cx43 proteins in B-cells, Wehi231 cells (which express endogenous Cx43) were transduced with Cx43T154A and sorted for EGFP expression as described previously (Chapters 2.3.2 and 2.5). Wehi231
Figure 3.7 Cx43T154A acts as a dominant negative of BCR-mediated cell spreading in Wehi231. A) Images of the contact site between cells and anti-IgM (BCR) coated coverslips taken on an Olympus Fluoview 1000 confocal microscope. Green=EGFP-fused WT Cx43 or Cx43T154A red=rhodamine phalloidin stained actin; merge = yellow. Scale bars represent 20 µm. B) Quantification of contact area between the cells and the anti-BCR coated coverslip. Error bars indicate standard error of the mean. Data is representative of three independent replicates in which >100 cells were quantified per sample. Asterixes (*) denote significant difference between cell types compared to untransfected cells as determined by P-value <0.05 by a student’s two-tailed, unpaired t-test calculated by Excel. Double asterix (**) indicates significant increase in contact area, as compared to decrease (*).
cells un-transduced or expressing WT Cx43 spread on anti-lgM coated glass coverslips (Figure 3.7). However, Wehi231 cells expressing Cx43T154A were inhibited from spreading, and thus the mutated Cx43 acted as a DN and prevented B cell spreading. While some (~5%) of cells exhibited protruberances at 30 and 120 min time points, roundness was not quantified due to insufficient numbers of cells exhibiting this phenotype as compared to J558µm3 cells (Figure 3.6). We next consider the mechanism by which this mutation influences Cx43’s effect on BCR-mediated cell spreading.

3.1.8 Rap1 activation is required for Cx43’s affect on BCR-mediated spreading in the J558µm3 B-cell line and is impeded by expression of Cx43T154A

Rap1 is a GTPase responsible for regulating cytoskeletal rearrangement involved in processes such as spreading in B-cells (Lin et al., 2009). We have previously shown that Cx43 is important for Rap1 activation, and in particular, that expression of the Cx43Δ246 mutation reduced the length of time that Rap1 remains activated following BCR or chemokine stimulation (Machtaler et al., 2011). While Cx43 is required for sustained Rap1 activation, we could not be certain that Cx43’s role in sustaining BCR-mediated Rap1 activation leads to the spreading seen in Cx43 overexpressing-transduced cells. Spreading of J558µm3 cells forced to express Cx43 is still much lower in magnitude than Wehi231 or A20 cells, both of which express Cx43 endogenously.
Figure 3.8 Rap1 activation is important for BCR-mediated spreading in J558μm3 plasmacytoma cells. BCR-mediated cell spreading of A) J558μm3 transiently transfected with constitutively active Rap (Rap1V12-FLAG) or B) J558μm3+Cx43-EGFP transiently transfected with RapGAPII-FLAG, which causes conversion of Rap1 into its inactive, GDP-bound form. Expression of Rap constructs was verified by anti-FLAG staining using an Olympus Fluoview 1000 confocal microscope using the 60x objective. Red=rhodamine phalloidin staining of actin; green=Alexa488 α-FLAG stained Rap1V12 (merge=yellow); blue=Alexa405 α-FLAG stained RapGAPII (merge=white). Contact area between the cell and anti-IgM (BCR) coated coverslip was quantified (C and D). Error bars represent standard error of the means. Scale bars represent 10 μm. Data representative of three independent replicates. Asterix (*) denotes significant difference between cell types as determined by P-value <0.05 by a student's two-tailed, unpaired t-test calculated by Excel.
To confirm that Cx43 expression influences spreading through Rap activation, rather than by increasing contact area non-specifically, J558μm3 cells stably expressing Cx43 were transiently transfected with FLAG-tagged RapGAPII which coverts Rap1 into its inactive, GDP-bound form (McLeod et al., 2002b). FLAG-tagged RapGAPII positive cells were stained with anti-FLAG and did not spread, indicating that the BCR-mediated spreading response of J558μm3 cells expressing Cx43 depends on Rap1 activation (Figure 3.8 B). In addition, expression of a FLAG-tagged Rap1V12, a constitutively active form of Rap1 (McLeod et al., 2002b), caused J558μm3 cells to spread to a similar magnitude as the spreading caused by Cx43 expression (Figure 3.8 A).

Since Rap1 activation is involved in BCR-mediated spreading, we hypothesized that the impaired spreading phenotype exhibited by B-cells expressing Cx43T154A may be a result of altered Rap1 activation, as is the case with Cx43 mutant Δ246 (Machtaler et al., 2011). A Rap1 activation assay was performed as described in Chapter 2.4.6 on J558μm3 cells expressing EGFP, Cx43, or Cx43T154A. In contrast to Cx43-expressing J558μm3, which showed sustained Rap1 activation up to 30 min, Cx43T154A or EGFP-expressing J558μm3 had a reduced level of active Rap1 at the 15 min time-point and almost no Rap1 activation after 30 min (Figure 3.9).
Figure 3.9 Rap1 activation in the J558μm3 B-cell line is less sustained when expressing Cx43T154A. Rap1 activation in J558μm3 cells expressing EGFP, Cx43-EGFP or Cx43T154A-EGFP. Cells were stimulated for the indicated times with 20 μg/ml of α-IgM (BCR) and activated Rap1 was precipitated using a GST-RalGDS fusion protein and total Rap1 detected by blotting with α-Rap (for detailed method see Chapter 2.4.6). Blot is representative of three independent replicates. (Data by May Dang-Lawson).
Cx43 influences Rap1 activation downstream of BCR crosslinking but it is not clear how. Both truncation of the CT tail (Cx43Δ246) and point mutation (Cx43T154A) that could cause permanently closed Cx43 channels resulted in a similar phenotype: decreased Rap1 activation at 15 and 30 min after BCR stimulation, and impaired spreading in response to BCR crosslinking. One possibility is that Cx43 influences B-cell spreading and the activation of Rap1 through channel activity by allowing entry or exit of some small molecule or ion essential for BCR signaling or cytoskeletal rearrangement, and that the CT region of Cx43 is important for regulating HC gating. The CT tail contains sites for phosphorylation which controls Cx43 trafficking, GJ assembly, conductance, and GJ turnover (Shin et al., 2001; Solan and Lampe, 2009a). The CT tail also contains binding sites for proteins that regulate GJ conductance (Lampe and Lau, 2004; Lin et al., 2001b; Solan and Lampe, 2009a), and may be able to conformationally interact with other regions of the Cx43 protein. For example, the CT has been proposed to influence GJ channel-gating by binding the intracellular loop (Ponsaerts et al., 2010). It remains to be determined whether the CT tail regulates HCs as it does GJ channels.

A second possibility is that the CT of Cx43 acts as a binding site for interactions with proteins either involved in the propagation of BCR signaling or cytoskeletal rearrangement. It is possible that the point mutation Cx43T154A causes a conformational change to the Cx43 structure specifically to the tail. To investigate this possibility we used two approaches: One was to create a Cx43 mutant with both the Cx43T154A point mutation and truncation of the cytoplasmic CT tail (Cx43T154AΔ246), and the second was to pharmacologically block the channel.
Figure 3.10 Involvement of the CT in non-radial spreading phenotype of Cx43T154A expressing cells. A) Cell spreading on α-IgM (BCR) coated glass coverslips. Images represent cell spreading at 30 minutes. One representative cell is enlarged showing the protrusions formed by cells expressing Cx43T154A-EGFP (arrow). Red=rhodamine phalloidin stained actin. Scale bars represent 10 µm. B) Quantification of the contact area between the cells and the coverslip. Error bars represent standard error of the mean. C) Percentage of cells exhibiting a non-radial spreading phenotype (arrow shown in A). Protrusions were counted if they exceeded a minimum length of half the cell diameter. D) Western blot analysis showing the expression of Cx43T154AΔ246 in the J558µm3 cell line. Membrane was stripped and re-probed for actin as a loading control. Data is representative of three independent replicates in which >100 cells were quantified per sample. Asterixes (*) denote significant difference between cell types compared to cells expressing WT Cx43 as determined by P-value <0.05 by a student’s two-tailed, unpaired t-test calculated by Excel. (**) denotes significant increase.
3.1.9 Removal of the cytoplasmic CT tail of Cx43T154A prevented non-radial B-cell spreading completely

A single round of site directed mutagenesis using the EGFP-fused Δ246 Cx43 construct from the Dr. Christian Naus’ lab (Bates et al 2007) as a template resulted in the generation of Cx43 mutant Cx43T154AΔ246 (Table 2.1). Cx43T154AΔ246 was introduced into J558µm3 cells, which were infected with supernatant collected from the retroviral packaging cell line BOSC23. Populations of Cx43T154AΔ246-EGFP expressing cells were selected for EGFP expression by FACS sorting and periodically re-sorted to maintain high levels of Cx43T154AΔ246-EGFP expression. Expression was confirmed by western blotting (Figure 3.10 D).

J558µm3 cells expressing Cx43T154AΔ246 did not spread and appeared similar to cells expressing either Cx43Δ246 or EGFP alone (Figure 3.10 B and C). This confirmed that BCR-mediated spreading was dependent on the Cx43 cytoplasmic CT tail and that the non-radial spreading phenotype of cells expressing Cx43T154A was not a result of closed HCs alone, but must be caused either by involvement of the tail in HC gating or disruption of a tail domain caused by this upstream mutation.
3.1.10 Preliminary experiments to test the effect of Cx43T154A on B-cell migration

BCR-mediated spreading was used as a readout for B-cell processes that rely on reorganization of the cytoskeleton in general. B-cell migration involves a polarized breakdown of the existing actin cytoskeleton to generate a leading front and formation of the lamellipodia (Ridley et al., 2003). This process has many similarities to those controlling B-cell spreading. Many of the same players become activated by signaling initiated by integrin engagement and by BCR stimulation, such as the master cytoskeletal regulator Rap1 (Lin et al., 2010).

Cx43 expression has been shown to affect the migration of neural crest cells, epithelial cell, fibroblasts, neural cell types, and B-cells, and appears to do so through influencing cytoskeletal organization and cell polarity (for review see (Matsuuchi and Naus, 2012)). The intermediate non-radial spreading phenotype exhibited by J558µm3 cells expressing Cx43T154A suggests that Cx43 may influence polarity of B-cells as well.

EGFP-fused Cx43T154A co-localized with actin in the protrusions formed by B-cells spreading non-radially on anti-BCR coated glass (Figure 3.6 A arrows). Cx43T154A localization was predictive of the dominant branch of the bifurcated leading edge of neurons migrating along radial glia (Elias et al., 2007), suggesting that Cx43T154A may have affects on cell polarity. It would be interesting to determine whether B-cell expressing Cx43 mutant Cx43T154A would exhibit defects in directional migration, in addition to BCR-mediated spreading.
Figure 3.11 Preliminary experiments for testing the effect of Cx43T154A on B-cell migration. Three different B-cell lines (A20, Wehi231, and J558µm3) were compared in the presence and absence of chemokine SDF-1 (CXCL12). A) Fluorescence microscopy showing tracks made by migrating cells. Red=rhodamine phalloidin stained cells (actin), blue=FluoroSpheres (Molecular Probes). Scale bars represent 30 µm. Error bars represent standard error of the means. Data is representative of two pilot experiments. B) Mean distance was quantified and asterixes (*) denote significant difference between untreated and SDF-1 treatment as determined by P-value <0.05 by a student's two-tailed, unpaired t-test calculated by Excel. C) Rap1 activation in J558µm3 cells expressing EGFP, Cx43-EGFP or T154A Cx43-EGFP (see Chapter 2.4.6). Cells were stimulated for the indicated times with 100ng/ml SDF-1/CXCL12. Data is representative of three independent replicates.
Our lab has previously shown that Cx43 expression increases B-cell adhesion, spreading, and migration (Machtaler et al., 2011). Migration was measured using two different assays: firstly a two-dimensional bead-clearing assay (see Chapter 2.6.5), and secondly a transwell assay that mimics transmigration and measures migration of cells across an endothelial monolayer, towards a chemokine source. We have yet to look at the role of Cx43 on B-cell polarization or at directional migration towards a chemokine source.

Cx43 expression improves the activation of Rap1 in response to stimulation of the BCR, integrin LFA-1, and also chemokine SDF-1/CXCL12 (Machtaler et al., 2011). Cx43T154A may be expected to cause defects in directional migration since expression of Cx43T154A resulted in less Rap1 activation than expression of WT Cx43 by J558µm3 cells (Figure 3.11 A). Although J558µm3 cells made an excellent GOF system to study the importance of Cx43 in BCR-mediated B-cell spreading, plasmacytoma cells are derived from highly specialized antibody-secreting plasma cells that have lost much of their ability to interact with cells and extracellular matrix and do not perform well in migration assays in vitro. Since Cx43T154A had a DN effect on WT Cx43 function in BCR-mediated cell spreading (Chapter 3.1.7), B-cell lines with endogenous expression may be used as alternatives to this cell line.

In order to determine the best cell line to use in a study of the effect of Cx43T154A on B-cell migration, three different cell lines were compared for their ability to migrate and their responsiveness to SDF-1. The A20 mouse B-cell lymphoma line migrated the furthest distance and was the most responsive to SDF-1 out of the cell lines tested (Figure 3.11 A and B). The
response of A20 in this assay makes them the best choice for investigating the effect of Cx43T154A in the future.

Chapter 3.1 in summary

Our finding that Cx43 was important for BCR-mediated B-cell spreading through a non-channel mechanism is consistent with a growing list of non-channel functions of the Cx proteins. We developed a dye-uptake assay of HC activity and showed that B-cells did not form HCs unless Cx43 was overexpressed (Section 3.1.2), and that blocking HC activity, using drugs had no effect on BCR-mediated B-cell spreading (Section 3.1.3). In addition, we predicted that the commonly used closed-channel mutant Cx43T154A may result in conformational changes to Cx43, that affect the CT domain particularly, in light of the necessity of this region for the production of non-radial projections on anti-BCR coated glass by expression of Cx43T154A (Section 3.1.9).

Because of the importance of the CT tail in Cx43’s influence on BCR-mediated B-cell spreading, as shown by both Cx43Δ246 and Cx43T514AΔ246 mutations, we next turned our attention to investigating the specific region responsible for the importance of this domain. A more detailed investigation of this region through mutation of specific residues will hopefully allow us to understand Cx43’s effect on cytoskeletal organization in B-cell processes that are important for their immune function and contribute to an understanding of Cx43’s non-channel functions in general.
3.2 The importance of tyrosine 265 of the Cx43 cytoplasmic CT tail for B-lymphocyte spreading

3.2.1 Rationale

Cx43 is phosphorylated in response to BCR stimulation (Guan et al., 1996; Machtaler et al., 2011), but in this system it is unclear if the phosphorylation occurs on serine, threonine, tyrosine residues or on all three. Over the past 15 years, the Cx43 literature has discussed the importance of both forms of phosphorylation, in particular in the role of these modified amino acids as potential binding sites for other proteins (Hervé et al., 2004; Palatinus et al., 2012; Shin et al., 2001), in regulating GJ formation (Shin et al., 2001; Solan and Lampe, 2009a), or in gating channel function (Lampe and Lau, 2004; Lin et al., 2001a; Solan and Lampe, 2009a). Given Steve Machtaler's previous work in the lab showing the importance of the Cx43 CT tail for BCR-mediated spreading, cell adhesion and migration, as well as the lab's historical interest in the importance of tyrosine phosphorylation of the BCR itself and downstream targets, we first explored the importance of tyrosine phosphorylation of the Cx43 tail.

The Cx43 cytoplasmic CT tail contains two tyrosine residues that could serve as docking sites for the Src-family protein kinases, and potentially phosphorylated them: tyrosine 247 (Y247) and tyrosine 265 (Y265) (Giepmans et al., 2001). Src kinase activity is required for Src binding to Cx43 as measured by Co-IP, and the presence of active Src is required for Y265 phosphorylation in vitro (Giepmans et al., 2001), therefore it seemed likely that Src kinase or
a Src kinase family member was responsible for some Cx43 phosphorylation and its subsequent ability to interact with Src. A model of Src kinase interaction with Cx43 has been proposed by Lin et al (Lin et al., 2001b). In this model, the SH3 domain of Src kinase binds to a proline-rich region of the Cx43 tail (located between aa 274-283), giving it the proximity to phosphorylate Y265. Following phosphorylation, Src could bind Y265 through its SH2 domain, facilitating subsequent phosphorylation of Y247. These phosphorylation steps are proposed to lead to closure of GJ channels (Lin et al., 2001b).

It remains unclear however, if Cx43 phosphorylation is important in B-cells. Considering our lab’s previous finding that the phosphorylation of Cx43 is a target of BCR signaling (Machtaler et al., 2011), we are interested in the Cx43 phosphorylation and its possible role in regulating the effect of Cx43 expression on B-cell spreading. This would make sense if the phosphorylated residues served as binding sites for cytoskeletal adaptors or other proteins involved in regulating cell shape and movement. Based upon historically well-documented studies of Cx43’s protein-protein interactions (Hervé et al., 2004), we decided to focus on the proposed Src kinase phosphorylation/binding site of Cx43: Y265 in our first detailed look at the function of the Cx43 tail.

Tyrosine phosphorylation is an initial step in propagating signaling after BCR stimulation, and downstream signaling activates Src kinase family members Lyn, Fyn, Blk and Lck in B-cells (for review, see (Defranco, 1997)). Src family kinases activated by the BCR may be responsible for the Cx43 phosphorylation in response to BCR stimulation. Activation of Src or Src kinase
family members expressed by B-cells could link the influence that Cx43 has on BCR-mediated spreading with Cx43 phosphorylation downstream of BCR stimulation.

To explore this possibility, we took two approaches, first, to make mutations in the tyrosines in the tail of Cx43 and see if this point mutation affected J558µm3 responses in one of our assays of cytoskeletal rearrangements. Second, we used biochemical methods including phospho-tyrosine-specific antibodies to see if BCR stimulation resulted in phosphorylated tyrosine (pY) on either the endogenous Cx43 expressed by Wehi231 B-lymphoma cells or Cx43-EGFP overexpressed by J558µm3. We then determined if mutation of an important tyrosine residue in the Cx43 CT tail affected the phosphatase sensitive Cx43 band-shift in response to BCR stimulation.

3.2.2 Expression of Cx43 cytoplasmic CT tail mutants in a B-cell line: J558µm3

Y265 was targeted because of its proposed role in Src kinase binding as a prerequisite for Y247 phosphorylation (Lin et al., 2001a). Tyrosine 265 was substituted with either phenylalanine (F) or aspartic acid (D) (Figure 3.12 A) by site directed mutagenesis (Chapter 2.2.6) using primer pairs (Table 2.1) containing a single base-pair substitution to mutate WT Cx43-EGFP in plasmid NAP2 (Bates et al., 2007) as a template for mutagenesis.

Cx43Y265F (F= phenylalanine in single aa code) tagged with EGFP and Cx43Y265D (D= aspartic acid in single aa code) tagged with EGFP were introduced into the J558µm3 cell line which lacks endogenous Cx43 expression and which had previously been transfected with the BCR (Dylke et al., 2007). Cells were infected with supernatant collected from the retroviral
Figure 3.12 Characterization of J558μm3 cells expressing Cx43Y265F and Cx43Y265D.
A) Schematic showing point mutation of tyrosine 265; one of two potential pY sites in the CT.
B) Western blot showing expression of Cx43Y265F and Cx43Y265D in the J558μm3 cell line stably transduced by retroviral infection (Blot was stripped and re-probed for actin as a loading control). C) Localization of EGFP-fused Cx43Y265F and Cx43Y265D at the plasma membrane of J558μm3 cells viewed using an Olympus Fluvview 1000 confocal microscope using a 60x objective (enlarged image shown on the left). Green=mutant Cx43-EGFP, blue=DAPI stained nuclei. Scale bars represent 10 μm. Data shown is representative of three experiments.
packaging cell line BOSC 23 (Chapter 2.3.2). Populations of Cx43Y265F or Cx43Y265D expressing cells were selected by isolating EGFP+ cells by FACS sorting (Chapter 2.5). Populations were periodically re-sorted to maintain high levels of Cx43 expression. Expression was confirmed by western blot analysis (Figure 3.12 B).

Expression of tyrosine mutants has been used to study tyrosine phosphorylation in the literature (Lin et al., 2001a). While Cx43Y265F has been shown to prevent Gj conductance, it did not disrupt the assembly of Cx43 into Gj plaques based on localization by immunofluorescence (Lin et al., 2001a). Expression of mutants Cx43Y265F and Cx43Y265D on the plasma membrane of J558µm3 was verified by confocal microscopy via fused EGFP tag (Figure 3.12 C), which we found to accurately predict surface expression by biotinylation (Chapter 3.1.2).

3.2.3 Phosphorylation of Cx43 in response to BCR-signaling

While the apparent increase in molecular weight of Cx43 in response to BCR stimulation was not due to phosphorylation of Cx43 tyrosine residues (Section 3.1.5), It remains possible that Y265 structurally affects Cx43 CT tail conformation and influences the availability of other sites for phosphorylation. To determine the importance of Cx43 residue Y265 for BCR-mediated phosphorylation of Cx43, J558µm3 cells expressing these mutants were stimulated with anti-IgM and then analyzed by SDS-PAGE and western blot (Chapter 2.4.2) to look for Cx43 band-shift. Mutation of Cx43Y265 did not abrogate BCR-mediated band-shift (Figure 3.13), suggesting that the importance of Y265 for BCR-mediated spreading can be accounted
**Figure 3.13** Mutation of Cx43 residue Y265 does not prevent band-shift in response to BCR stimulation. Western blot showing a phosphate-specific shift in Cx43 molecular weight in response to stimulation with 20 µg/ml of α-IgM (BCR) for the time-course shown. Cx43 band shift was abrogated by treatment of samples with 3 units/30 µg calf intestinal phophatase (CIP). Membrane was stripped and re-probed for actin as a loading control. Data shows one pilot experiment.

for by a mechanism other than phosphorylation, or that the phosphorylation of Cx43Y265 is at too low a level or is too transient to be detected by our methods. Possible reasons why phosphorylated Y265 may not have been detected even if it was present will be discussed in Chapter 4. Another possibility is that the phosphatase-sensitive Cx43 band shift was caused by phosphorylation of serine or threonine residues. The lysates of J558µm3 cells expressing Cx43 will be shipped to the laboratory of Dr. Paul Lampe (Fred Hutchinson Cancer Research Centre, Seattle) for western blotting and detection using precious monoclonal serine-specific anti-Cx43 antibodies.
Figure 3.14 Tyrosine phosphorylation is not responsible for Cx43 band-shift in response to BCR signaling. A) Schematic of Cx43 CT showing potential tyrosine phosphorylation sites and specificity of antibodies used in western blot analysis of Wehi231 or J558µm3+Cx43-EGFP cells stimulated with soluble α-IgM (BCR). Cells were lysed after stimulation and either immunoprecipitated with α-Cx43 (Sigma) (C and E for Wehi231 and J558µm3+Cx43-EGFP respectively), or loaded without purification (B and D for Wehi231 and J558µm3+Cx43-EGFP respectively). Blots were probed with α-pY: 4G10 (Cell Signaling), α-Cx43 (Fred Hutchinson), or α-pY265 Cx43 (Solan et al 2008). Data is representative of one pilot experiment for Wehi231 and two experiments for J558µm3+Cx43-EGFP.
Since BCR-stimulation leads to Cx43 phosphorylation (Machtaler et al., 2011), and since mutation of Y265 prevents BCR-mediated cell spreading (see Section 3.1.4), we hypothesized that this tyrosine residue, located in the Cx43 CT region, may be an important phosphorylation target downstream of BCR signaling. The antibody 4G10 was used to detect phospho-tyrosine (pY) by western blot in Wehi231 or Cx43-expressing J558µm3 cells that had been stimulated with anti-IgM (Figure 3.14 B-D upper panels) as well as with phospho-tyrosine antibodies from BD Biosciences (#610009) and Cell Signaling (#9411) (Data not shown).

Since Src kinase family members phosphorylate tyrosine residues found in the ITAMs of Igα and β following BCR stimulation (Defranco, 1997), BCR signaling results in high levels of tyrosine phosphorylation that obscure the ability to discern a band of Cx43’s molecular weight with certainty (Figure 3.14 B and D, upper panels). Stimulation of Cx43-expressing J558µm3 cells resulted in a lower level of total tyrosine phosphorylation compared to Wehi231 cells (Figure 3.14 D). Since the amount of tyrosine phosphorylation in response to BCR stimulation made it difficult to distinguish single proteins accurately based on molecular weight, Cx43 was immunoprecipitated using an antibody against the cytoplasmic CT tail (Sigma). A second antibody against the amino-terminal domain of Cx43 (Fred-Hutchinson) was used to detect Cx43 in the pull-down and total cell lysate, which confirmed presence of Cx43 as well as characteristic band-shift in response to stimulation (Figure 3.14 B and D, lower panels). No band at Cx43’s molecular weight was detected with 4G10 (Figure 3.4 C and E), nor with phospho-tyrosine specific antibodies for Cx43 (Solan and Lampe, 2008).
3.2.4 Importance of Cx43Y265 for BCR-mediated B-cell spreading

BCR-mediated cell spreading is important for Ag gathering and B-cell activation, but this process is also commonly used as a read-out for processes that depend upon rapid cytoskeletal rearrangement (Lin et al., 2008b). Since the Cx43 cytoplasmic CT tail has been found to be necessary for B-cell spreading via expression of a truncated form of Cx43 (Machtaler et al., 2011), we decided to take a more detailed look at a tyrosine residue (Y265) of the tail in order to better understand the mechanism of Cx43’s affect on these B-cell processes.

Neither J558µm3 cells expressing Cx43 mutant: Cx43Y265F or Cx43Y265D spread on anti-IgM coated glass coverslips (Figure 3.15 A, quantified in B). The contact area of mutant-expressing cells was not significantly different from Cx43Δ246 expressing cells which do not spread effectively by 30 min, whereas WT Cx43 expressing cells spread normally, and to a significantly greater extent than, Cx43Δ246, Cx43Y265F and Cx43Y265D expressing cells based on a two-tailed t-test using a 95% confidence interval.

This shows that tyrosine residue 265 of the Cx43 tail is required for effective B-cell spreading and highlights a single residue, Y265 as the possible cause for the defect in spreading observed with Cx43 mutant Δ246. We are still uncertain of the mechanism underlying Y265’s importance in B-cell spreading however; therefore the next step will be to determine whether Y265 acts as a phosphorylation site for Src kinase or another kinase. These questions form the basis of a new graduate student’s project in the Matsuuchi lab.
**Figure 3.15 Y265 is necessary for BCR-mediated spreading in the B-cell line: J558µm3.**

A) Confocal images of spreading of J558µm3 cells expressing Cx43 tyrosine mutants Y265D (D=Aspartic Acid) and Y265F (F=Phenylalanine), and the CT truncation mutant (ΔCT at aa 246=Δ246) on 40 µg/mL α-IgM coated coverslips (red=rhodamine-phalloidin stained actin, green=WT or mutant Cx43-EGFP, merge=yellow). B) Spreading was quantified by measuring the contact area between cells and the glass coverslips after the indicated minutes after BCR stimulation by crosslinking with α-IgM. Images were obtained using an Olympus Fluoview1000 confocal microscope using a 60x objective. Scale bars represent 20 µm (10 µm for enlargement). Data is representative of three experiments in which >100 cells were quantified per sample. Asterix (*) denotes significance between cell types compared to WT Cx43 as defined as P-value >0.05 by a student's two-tailed, unpaired t-test calculated by Excel.
Chapter 3.2 in summary

A single tyrosine residue, Y265 located in the cytoplasmic CT tail domain of Cx43 was found necessary for BCR-mediated B-cell spreading of the J558μm3 cell line, as shown by expression of two different mutant constructs containing point mutations at this aa (Cx43Y265F and Cx43Y265D) (Section 3.2.4). Since this residue is a target of phosphorylation in other systems, Cx43 phosphorylation was measured in response to B-cell stimulation with antibodies used to crosslink the BCR. BCR stimulation lead to a phosphatase-sensitive increase in Cx43 MW by gel-electrophoresis (indicating phosphorylation), but it is still unclear which target aa of Cx43 are responsible for this shift. Expression of Cx43Y265F did not prevent BCR-mediated Cx43 MW shift. However the effect of single amino acid substitution at this site may have been masked by phosphorylation elsewhere in the Cx43 protein, or difficult to detect with the methods used.
CHAPTER 4
DISCUSSION

B-lymphocytes are key players of the adaptive immune response since they contribute to both humoral and cellular immunity. B-cells act as APCs by displaying peptides internalized through the BCR on MHC II molecules to activate helper T-cells. As well, B-cell activation leads to their proliferation and differentiation into plasma cells that secrete antibodies that are highly effective at neutralizing and opsonizing pathogens and toxins. B-cell activation depends upon the likelihood of any given B-cell coming into contact with an Ag that matches its unique BCR-specificity. Therefore a high degree of motility and migration are essential for naïve B-cells to enter and exit the vasculature, survey inflamed tissues, and move within different niches found in lymphoid organs such as the spleen and lymph nodes as well as in the bone marrow. While it is clear that rearrangements of the cytoskeletal architecture of B-lymphocytes must be regulated in order to orchestrate these cellular events, much remains to be discovered about how sequential breakdown and reformation of the cytoskeleton is controlled and how regions of the cell are targeted differentially.

It is attractive to propose that other families of molecules work in concert with signaling receptors to regulate these processes. In fact, the GJ protein family, the Cxs, share many structural similarities with the tetraspanin protein family, which have no intrinsic signaling activity, but have been shown to be important for the polarization, motility and migration of various cell types (For review see (Kameritsch et al., 2011; Maecker et al., 1997; Matsuuchi and Naus, 2012; Olk et al., 2009)).
In B-lymphocytes, Cx43 knockdown using an shRNA strategy, was shown to reduce in vitro spreading in response to BCR-crosslinking in the Wehi231 murine lymphoma cell line which was derived from B-cells at a naïve stage of development (Machtaler et al., 2011) and to decrease adhesion to endothelium, impair transmigration and motility on fibronectin, as well as inhibit extravasation through endothelial cell layers (Bruzzone et al., 2003). Expression of Cx43 in B-cells is dependent on the stage of differentiation and is lost in terminally differentiated plasma B-cells (Machtaler et al., 2011). As well, differentiated plasma cells don't express surface BCR, instead secreting antibodies exclusively. This observation is meaningful since B-lymphocytes in earlier stages of differentiation interact with, and move across and through cells in the bone marrow, lymph node and blood vessels as they travel throughout the body during normal development and during immune responses. In contrast, terminally differentiated B-cells whose job is to secrete antibodies no longer need to interact with other cells and have less need to be actively motile.

Nevertheless, this difference in the properties of B-cells at different stages of development has been exploited in the studies in Machtaler et al., (Choi, MSc thesis, UBC, 2012) and in this thesis. The terminally differentiated, murine plasmacytoma B-cell line J558, normally secrete IgA and it has been immortalized in tissue culture and used for over 45 years as a tool to study B-cell function and antibody production. A version of J558, has been previously transfected with the membrane bound BCR (mIgM, Igα and Igβ), resulting in a tissue culture cell line called J558μm3 but has been used to study BCR signaling (Hombach et al., 1988). J558μm3 with cell surface BCR, does not normally spread (McLeod et al., 2004) in response to
BCR-stimulation, however overexpression of Cx43-EGFP was sufficient to restore BCR-mediated spreading in these cells (Machtaler et al., 2011), a phenomenon that normally occurs after BCR signaling in immature and mature B-cell lines (ie, Wehi231 and A20).

Expression of a mutated form of Cx43 with the CT domain truncated (ΔCT at aa 246, =Δ246) was not sufficient to cause J558μm3 spreading or adhesion to endothelium (Machtaler et al., 2011), suggesting that there are regions of the CT domain of Cx43 important for these effects. The exact role of the CT domain is unclear and there has been much speculation as to why this occurs.

While the CT domain of Cx43 was shown to be important for B-cell adhesion and spreading (Machtaler et al., 2011) and is responsible for the effects of Cx43 on migration of other cell types (Bates et al., 2007; Behrens et al., 2010; Francis et al., 2011b), the mechanism responsible for its importance is currently being explored in a number of labs, including ours. Many explanations of the role of the Cx43 CT domain in cellular migration have focused on its potential binding to the cytoskeleton or to cytoskeletal adaptors, perhaps by phosphorylated residues in the CT. However, the majority of the phosphorylation sites in the CT region and reported protein interactions of Cx43 have been shown to regulate GJIC channel activity (Hervé et al., 2004) and do not yet specifically target a cytoskeletal adaptor binding site. Although most CT truncations do not appear to impair Cx43 trafficking or formation of GJ plaques, the Δ246 mutation resulted in reduced GJ conductance in HeLa (Behrens et al., 2010) and C6 glioma cells (Lin et al., 2003), suggesting that expression of mutant Cx43 missing the CT domain after aa 246, may have effects through influencing channel conductance.
The mechanism of channel gating is complex and may involve all three cytoplasmic domains (Evans and Martin, 2002) which may act as voltage sensors, pH sensors, or sites of phosphorylation that effect channel function. In particular, serine and tyrosine residues found in the CT domain act as sites for phosphorylation by MAPK, and Src kinases. Phosphorylation of the CT domain of Cx43 has been shown to regulate GJ conductance and may do so 1) by targeting the Cx/GJ for internalization and reducing overall levels of the channel on the cell surface, 2) by changing the conformation of the protein such that channel activity is altered/blocked, or 3) by resulting in protein-protein interactions that influence the accessibility of the GJ channel’s pore. While few studies have investigated the gating of HCs due to their difficulty to activate and measure, it is likely that the CT domain may regulate both classical GJ channel conductance and HC conductance in a similar way (Bukauskas et al., 2005). BCR stimulation results in the phosphorylation of Cx43 in Wehi231 B-cells (Machtaler et al., 2011) and in other B-cell lines that have been subsequently tested (unpublished data). One way that phosphorylation of Cx43 could influence B-cell spreading, is through changing the conductance of Cx43 channels.

Although, many of the studies done to investigate the role of Cx43 on cellular migration have concluded that its effects are channel-independent, channel blocking strategies are typically only confirmed by their ability to block GJIC, leaving HC function untested. While B-cell spreading and migration were assayed on coated glass or plastic (ruling out the possibility of GJ formation and cell-cell communication), there is a possibility that Cx proteins form HCs that can open under certain conditions that promote activation. HCs can facilitate the release into the extracellular space of small molecules and ions that have been shown to have
autocrine and paracrine actions through purinergic signaling (Baroja-Mazo et al., 2012), and this may contribute to T-cell activation through the amplification of TCR signaling (Mendoza-Naranjo et al., 2011), and to cross-presentation of Ag peptides to professional APCs (Neijssen et al., 2005). The possible involvement of HCs at immune synapses makes it important to determine if Cx43 affects BCR-mediated spreading through HC function, since spreading contributes to formation of the B-cell-APC immune synapse (Fleire et al., 2006). The possibility that the CT domain of Cx43 may regulate the conductance or selectivity of HCs caused us to follow up on the finding that the CT domain of Cx43 is important for B-cell spreading by determining 1) if B-cells make HCs and 2) if the activity of Cx43 HCs contributes to BCR-mediated B-cell spreading. The effect of Cx43 channel-function on B-cell spreading was addressed using two different approaches: a pharmacological strategy to block HCs, and a genetic strategy using a mutant Cx43 that does not form functional GJ channels.

BCR-mediated cell spreading was unaffected by treatment with agents that block Cx HCs (La$^{3+}$), Panx HCs (Pbn), or both (CBX) (Figure 3.1). These results were found for B-cell lines expressing endogenous Cx43 (A20 and Wehi231 cell lines) that did not form HCs upon activation by the removal of divalent cations. The regulation (removal) of cations, which is a typical HC activator and effective for opening HCs in astrocytes, was used as a positive control (Figure 3.2). However the J558µm3 cell line overexpressing Cx43-EGFP was found to exhibit a high level of HC activity even in media containing concentrations of Ca$^{2+}$/Mg$^{2+}$ that are sufficient to block HCs normally. This means that the divalent cation containing solution (DCFS -), as opposed to divalent cation free solution (DCFS +), prevented dye-uptake by astrocytes. This dye-uptake was specific to Cx43 HCs and can be inferred from the blocking of
dye uptake by CBX pre-treatment and by the lack of HC activity detected in J558µm3 overexpressing EGFP alone. High basal HC conductance is typical of cells overexpressing Cxs. In fact, overexpression is one of the few experimental systems where Cx HCs can be effectively evaluated since HCs typically exist in a constitutively closed state and are only opened by specific activating conditions (Bukauskas et al., 1995). The spreading of J558µm3 cells expressing Cx43 when treated with HC blockers (Figure 3.1) suggests that BCR-mediated spreading is independent of HC activity and that spreading is not a product of the increased HC activity exhibited by Cx43-expressing J558µm3 cells.

Given the most likely conclusion (based upon the lack of effect that HC blocking agents had on B-cell spreading (Figure 3.1), that Cx43 influences BCR-mediated B-cell spreading by a channel-independent mechanism, it was initially surprising that expression of Cx43 closed-channel mutant Cx43T154A resulted in an unusual spreading phenotype by both Cx43-null J558µm3 cells (Figure 3.6) and Cx43+ Wehi231 cells (Figure 3.7). Unlike WT Cx43-expressing cells which spread radially on anti-BCR coated glass, populations of B-cells overexpressing Cx43T154A frequently formed a single membrane protrusion per cell that extended from the cell surface and attached to the coated glass at the tip of the protrusion (Figure 3.6 A arrows). This phenotype did not appear to be due to a reduction in cell surface expression of the Cx43 mutant (Figure 3.3). In addition, EGFP fusion with Cx43 has been characterized in other papers in the literature (Laird et al., 2001) and did not appear to prevent expression of Cx43 at the cell surface in our J558µm3 cell expression system (Figure 3.5). Therefore proper trafficking and cellular localization of Cx43T154A probably does not contribute to the non-radial spreading phenotype observed.
The Cx43T154A mutation could act as a DN by forming heteromeric HCs with WT Cx (Good et al., 2011). This is consistent with our finding that the expression of Cx43T154A prevented radial spreading and in some cases, caused the formation of unidirectional membrane protruberances in the Wehi231 cell line that expresses Cx43 endogenously similar to those unidirectional processes found in Cx43T154A-expressing J558µm3 plasmacytoma cells (Figure 3.7). These processes originated from the surface of the cell not in contact with the antibody-coated coverslip which could explain why only ~5-50% of cells exhibited this phenotype. The formation of membrane protrusions on the dorsal side of spreading cells that were long enough to reach the coverslip may result from an inability of Cx43T154A expressing cells to polarize into a spreading side with actin polymerization and branching and a non-spreading side without. The formation of actin-rich membrane protuberances away from the site of BCR-stimulation may demonstrate Cx43’s importance in regulating polarized actin dynamics in B-cells.

Since BCR-mediated B-cell spreading, adhesion and migration share in common their reliance on reorganization of the cytoskeleton, we next looked at the activation of the master regulator of the actin cytoskeleton in B-lymphocytes (among other cell types): the Rap1 GTPase. Rap1 has emerged as a regulator of the activities of actin binding proteins in B-cells (McLeod and Gold, 2001), and has been shown to be involved in B-cell adhesion, spreading, migration, and invasion by the expression of mutated constructs (Lin et al., 2009; Lin et al., 2008b; McLeod et al., 2002a; McLeod et al., 2004). Cx43 expression has been linked to sustained activation of Rap1 since shRNA knockdown of Cx43 resulted in less Rap1 activation
in the Wehi231 B-cell line and overexpression of Cx43 resulted in more sustained Rap1 activation in the Cx43-null B-cell line J558µm3 (Machtaler et al., 2011).

Forced expression of the Rap1 specific GAP (RapGAPII) in J558µm3 cells stably transfected with WTCx43 was sufficient to prevent BCR-mediated spreading on anti-BCR coated glass coverslips (Figure 3.8 B and D), suggesting that Cx43 expression influences spreading through a Rap1-dependent pathway. In addition, the overexpression of a constitutively active form of Rap1 (Rap1V12) caused J558µm3 cell spreading even in the absence of Cx43 (Figure 3.8 A and C). These results provide strong evidence for a mechanism of Cx43 involvement where expression of this GJ protein influences a pathway leading from BCR-signaling to Rap1 activation.

Since the expression of mutant Cx43T154A altered BCR-mediated spreading (Figures 3.6 and 3.7) we looked to see if Cx43T154A expression also altered Rap1 activation in response to BCR-stimulation and found that the expression of Cx43T154A had a similar effect on Rap1 activation as truncation of the CT domain did. Cells expressing Cx43T154A or EGFP alone produced an initial spike in Rap1 activation following BCR-stimulation, but much less active Rap1 was detected at later time-points compared to cells expressing WT Cx43 (Figure 3.9).

One possibility for this is that both the CT domain and the channel function of Cx43 are essential for the activation of the Rap1 GTPase, either through two separate mechanisms, or because the CT domain is involved in channel gating. It seemed unlikely however, that the tail of Cx43 influenced BCR-mediated B-cell spreading through gating Cx43 channel activity given
the lack of effect that channel-blocking drugs has on B-cell spreading (Figure 3.1). Another possibility to explain the similarities between Cx43Δ246 and Cx43T154A on Rap1 activation is that the Cx43T154A mutation results in a conformational defect to Cx43 tertiary structure of the cell surface Cx43-containing connexons that alters the functionality of the CT domain.

The Cx43T154A point mutation was chosen out of a larger mutagenesis study as a tool to study the role of Cx43 channel function because of the ability of this mutant to traffic to the plasma membrane and form GJ plaques, while inhibiting dye-transfer between expressing Xenopus oocytes (Beahm et al., 2006). However the effects of the Cx43T154A point mutation to the tertiary structure of Cx43 are unclear and could result in protein miss-folding due to substitution of a non-polar aa in place of a polar aa in the hydrophilic channel-lining TM3 domain. HCs made of Cx26 mutated at an analogous conserved threonine residue: T135A, resulted in less tightly packed hexomers in GJ plaques and increased dissociation of oligomers in detergent (Beahm et al., 2006). These observations seem to indicate that Cx43T154A or Cx26T135A containing proteins may have a slightly altered conformation from WT Cx43 resulting in instability and altered packing of mutant-containing hexamers/HCs.

While the mechanism causing the mutated Cxs' DN effects were originally proposed to be due to reduced communication by heteromeric channels (Willecke et al., 1999), an alternative interpretation of the data suggests that mutated Cxs could have DN effects on WT Cx proteins in channel-independent ways as well. For example, Cx37T154A acted as a DN on the channel-independent influence of Cx37 slowing proliferation (Good et al., 2011). If the CT region of Cx43 is involved in protein-protein interactions leading to Rap1 activation and B-cell
Figure 4.1 A model showing how the CT domain of Cx43 mutants may have a dominant negative effect on B-cell spreading by interfering with WT Cx43 by forming heteromeric hemichannels. Cxs (shown as cylinders) oligomerize into hexameric HCs prior to trafficking to the cell surface. Heteromeric HCs can form in B-cells that have been transfected with Cx43 mutants (mutation of threonine T154 to alanine shown in green; truncation of the CT at Δ246 shown in purple), but which also express endogenous WT Cx43 (shown in orange). The cytoplasmic C-terminal (CT) tail of Cx43 is necessary for BCR signaling (BCR signaling=red arrow; thickness of the arrow indicates relative effectiveness of signaling), leading to B-cell spreading (tan colored cells below) and is predicted to interact with either an effector downstream of the BCR signaling cascade, or an adaptor of the cytoskeleton (red circle). The Cx43 point mutant T154A is predicted to cause a conformational defect to the CT tail, resulting in reduced ability to bind interacting proteins and this results in the generation of long membrane protuberances instead of radial spreading when plated on anti-BCR coated glass.
spreading downstream of BCR-signaling, then one possibility is that oligomers containing Cx43T154A are altered in their ability to influence these processes by the presence of the proteins containing mutations. For example, the CT tails of WTCx43 that are exposed in the cytoplasm could interact with other cytoplasmic proteins might be interfered with by the presence of Cx43T154A tails that could be miss-folded and in close proximity (Figure 4.1). This could result in the increased space between HCs as well as the decreased stability of oligomers made up of Cx43 containing the T154A mutation, similar to the proposal for Cx26 containing the T135A mutation (Beahm et al. 2006).

Structural defects in the CT domain could result in the DN effect of Cx43T154A expression if heteromeric channels are formed between mutant and WT Cx43. In addition, altered conformation of Cx43 CT domains could also explain why B-cells expressing Cx43T154A extend unidirectional protuberances in many cases (Figure 3.6 A arrows) instead of failing to spread at all. It is possible that an intermediate amount of protein interaction with defective CT tails of Cx43 is still able to occur and result in spreading as shown by increased contact area of cells plated on anti-BCR (Figure 3.6 B) although the cellular polarization or ability to sustain the spreading response until radial spreading is achieved is altered by this point mutation. It would be interesting to determine if truncation of the CT domain can act as a DN in Wehi231 cells, which would support this hypothesis. Additional truncation of the CT domain of Cx43T154A (Cx43T154AΔ246) completely prevented BCR-mediated spreading of J558µm3 and formation of membrane protuberances (Figure 3.10), emphasizing the importance of the CT domain of Cx43 for B-cell spreading and demonstrating that the protuberances formed by Cx43T154A-expressing cells is not due to the effect of this mutation.
on channel activity alone, in which case Cx43T154AΔ246–expressing cells could be expected to exhibit the same phenotype.

Cx43T154A is by far the most well used mutant to rule out channel formation as the mechanism behind functions of Cx proteins owing to its extensive characterization and DN effects. Recently, an alternate closed-channel Cx43 mutation: Cx43Y17S was used in a comparison with WT Cx43 to rescue migration in MEFs isolated from Cx43−/− mice (Francis et al., 2011b). This mutation was identified from a study of patients with ODDD (a human syndrome caused by mutations in the Cx43 gene: GJA1 (Paznekas et al., 2003)). Since this mutation is located in the NT of Cx43, further away from the CT domain, it is more likely that this mutation would be free of the kinds of conformational defects to the CT region that we suspect may be present in Cx43 containing the mutation Cx43T154A. This mutant may make an alternative genetic tool for differentiating the role of Cx43 in forming channels from the role of the Cx43 CT domain to act as a scaffold for protein interactions. Preliminary experiments are underway in the lab but it is too premature to comment at this time.

Experiments in our lab show that BCR signaling leads to a time-dependent and signaling-dependent increase in Cx43 MW by gel electrophoresis (Machtaler et al., 2011). The sensitivity of these bands to decreases in MW after digestion with calf intestinal phosphatase (CIP), which cleaves off all phosphates, suggests that BCR signaling leads to phosphorylation of Cx43. Cx43 has been shown in-vitro and in-vivo to be phosphorylated at multiple serine and tyrosine residues (Solan and Lampe, 2009a). These phosphorylation events may change the conformation of Cx43’s structure or change its ability to interact with adaptor proteins. Cx43
phosphorylation has been studied mostly for its effect on GJ gating, but phosphorylation could regulate non-channel functions as well.

Unlike WT Cx43, the mutant Cx43T154A did not appear to increase its MW to the same extent as WT Cx43, suggesting that its phosphorylation is altered upon BCR-stimulation (*data not shown*). These results provide further support for the idea that Cx43T154A could cause a conformational defect to other regions besides the pore, since the CT domain is the only region of Cx43 known to be phosphorylated (Solan and Lampe, 2009a). These results suggested to us that phosphorylation of the CT domain of Cx43 in response to BCR-signaling, may change the ability of Cx43 to interact with proteins involved in the activation of Rap1 and B-cell spreading and will be further studied by future graduate student’s in the lab.

Given the importance of tyrosine phosphorylation in response to BCR-signaling (Gold et al., 1990) as well as the recruitment of Src family kinases such as Lyn, Fyn, Lck and Blk by BCR activation (Gold et al., 1990), we decided to investigate the potential of tyrosine residues found in the Cx43 CT domain to act as targets of phosphorylation, that could be important for BCR-mediated B-cell spreading. Two tyrosine residues (Y265 and Y247) found in the Cx43 CT have been shown to be targets of v-Src phosphorylation and Y265 is proposed to be bound by v-Src following phosphorylation (Lin et al., 2001a). These tyrosines are reported to be phosphorylated sequentially, therefore the first of them, Y265, was chosen as our target, being both the first of the two tyrosines to be phosphorylated and because of its potential to act as a binding site for v-Src through its SH2 domain (Lin et al., 2001a).
Mutation of Y265 did not prevent CIP-sensitive band shift of Cx43 in response to BCR stimulation (Figure 3.12). In addition, tyrosine phosphorylation of Cx43 was not detected in response to BCR-stimulation using phospho-tyrosine specific antibodies (Figure 3.13). One possibility is that Cx43 is abundantly phosphorylated at serine or threonine and more rarely at tyrosine residues. Another is that phosphorylation at Y265 is not sufficient to change the mobility of Cx43 by gel electrophoresis since the addition of a single inorganic phosphate group (~80 kDa) can not account for the 2-4 kDa shift of Cx43 between P0, P1 and P2 states. Lastly, a third possibility is that phosphorylation of Y265 only occurs on a subset of Cx43 in the cell and that the detergent used to isolate Cx43 does not retrieve a sufficient enough pool of Cx43 phosphorylated on tyrosine residues to be sensitive to differences between WT and mutant forms.

Many experiments looking at Cx43 phosphorylation, and especially studies of Cx43 and v-Src interaction, have used GST-fusion proteins and overexpression of both Cx43 and v-Src, or constitutively active forms of v-Src (Filson et al., 1990; Giepmans et al., 2001; Kanemitsu et al., 1997; Lin et al., 2001a, b; Loo et al., 1995). Overexpression of Cx43 in our system, may overload the capacity of endogenous Src family kinases to phosphorylate Cx43 resulting in an undetectable pool of Cx43 with phosphorylated tyrosine residues. This overexpression would be consistent with the increased ER aggregate of Cx43-EGFP found in transduced cells (Machtaler et al., 2011), and it would also explain the basal HC activity of J558um3 cells expressing Cx43 (Figure 3.2). Normally HCs are very tightly regulated and exist in a closed conformation (Bukauskas et al., 1995). Phosphorylation of Cx43 has been shown to decrease GJ channel conductance, and while phosphorylation of Cx43 has been less documented as a
regulator of HC conductance, HCs may be regulated by similar mechanisms. If overexpressed Cx43 overloads the capacity of endogenous kinases to regulate Cx43 gating and turnover then this could explain the high basal activity by these cells.

While we could not show that tyrosine phosphorylation accounts for the Cx43 shift in molecular weight following BCR-stimulation, point mutation of Y265 to both phenylalanine (F) or aspartic acid (D) prevented spreading of expressing-J558µm3 cells (Figure 3.14), highlighting the importance of this residue for BCR-mediated spreading. Mutation of Y265 may alter Cx43 function by interfering with CT conformation in some way that is independent of phosphorylation, or which prevents phosphorylation at other sites. Alternately, phosphorylation of Y265 may be required for B-cell spreading but our biochemical methods of detection may not be sensitive enough to detect the loss of a single inorganic phosphate due to this mutation, in light of the many other phosphorylation sites in the CT tail which may mask any MW changes that tyrosine phosphorylation would cause.

HC activity did not influence spreading, so the question remains how phosphorylation of Cx43 affects BCR-mediated B-cell spreading by a non-channel mechanism. Phosphorylation could change the conformation of Cx43 thereby influencing its interactions with adaptor proteins by making binding sites more accessible, or less accessible. There are two ways in which Cx43 could influence BCR-mediated B-cell spreading through interactions of its CT domain with other proteins. One is that Cx43 acts as an adaptor with the cytoskeleton, and the second is that Cx43 influences BCR signaling directly by binding to effectors involved in the BCR-signaling cascade.
The cytoskeleton is often linked to the plasma membrane by transmembrane proteins and their adaptors at junctions. GJ protein Cx43 contains binding sites in its CT tail for tubulin (aa 234-243) as well as putative domains that may bind adaptors of the actin cytoskeleton such as the cortactin homologue HS1 (Hao et al., 2004; Van Rossum et al., 2005; Vitale et al., 2009) and the drebrin homologue HIP-55 (Butkevich et al., 2004; Ensenat et al., 1999). Cx43 also contains a PDZ binding domain that has been shown to bind ZO-1 in other cell types (Toyofuku et al., 1998), however, B-cells do not express ZO-1 or make tight junctions, and this region of the Cx43 CT tail is impeded by the fused EGFP tag in our expression system. Loss of Cx43 expression or overexpression are often linked with changes to the cytoskeleton and cellular morphology. The influence of Cx43 on cytoskeletal dynamics would explain how its expression influences processes as diverse as B-cell adhesion, spreading, motility, and migration, which share in common a requirement for cytoskeletal rearrangement.

Cx43 has been shown to influence the migration of many cell types (Kameritsch et al., 2011; Matsuuchi and Naus, 2012; Olk et al., 2009) and appears to do so through the effects of Cx43 on cellular morphology and the ability of cells to form polarized protrusions of the membrane; probably due to reorganization of the underlying cytoskeleton. In addition to its importance on mediating dynamic cell processes like spreading and migration, the cytoskeleton also provides support to the plasma membrane and can restricts the mobility of embedded proteins through attachment to membrane proteins with cytosolic domains capable of binding cytoskeletal proteins or their adaptors (Kusumi and Sako, 1996). There are two ways that Cx43 scaffolding with the cytoskeleton could influence BCR-mediated
spreading: 1) By stabilizing cytoskeletal dynamics during actin polymerization and branching leading to cell spreading, by linking the cytoskeleton to the plasma membrane (Figure 4.2 B i), or 2) By influencing the composition or stability of plasma membrane micro-domains through stabilization of the cortical actin cytoskeleton underlying the plasma membrane through a picket-fence mechanism (Figure 4.2 B ii).

Changes to cellular morphology and motility are achieved by combining adhesion with cytoskeletal reorganization. B-lymphocytes adhere to many cell-types: stromal cells during development (Hardy et al., 1991; Simmons et al., 1992), endothelial cells during transmigration (Carlos et al., 1990; Shimizu et al., 1992), and follicular dendritic cells within the spleen and lymph nodes (Koopman et al., 1991; Kosco et al., 1992); as well as to deposited extracellular matrix proteins like fibronectin (Chan et al., 1992; Stupack et al., 1991). In addition, it has been shown in vitro using an artificial lipid bilayer to model an APC, that B-cells spread in response to BCR-crosslinking (Fleire et al., 2006). B-cells bind to other cell types and to the extracellular matrix via adhesion proteins expressed on their cell surface. Adhesion proteins classically include members of the integrin, cadherin, selectin, and immunoglobulin superfamilies (Albelda and Buck, 1990; Springer, 1990), although evidence shows that the Cx family of proteins may also produce cell-cell adhesion through the disulfide bonds formed between adjacent HCs during Gj formation (Cotrina et al., 2008; Elias et al., 2007; Yamane et al., 1999) in addition to their more well-known role in cellular communication.
Figure 4.2 A model showing how Cx43 influences BCR-mediated Rap1 activation and B-cell spreading. A) BCR (green) crosslinking by anti-BCR or Ag (red dot) results in the recruitment of Src family kinases (such as Lyn, pink oval; and Syk, purple oval) which phosphorylate tyrosine residues of the BCR to initiate BCR signaling (blue arrows). BCR signaling leads to phosphorylation of Cx43 (orange), as well as activation of Rap1 (red rectangle) which causes actin (red lines) severing leading to increased mobility of BCRs that results in B) formation of BCR micro-clusters that generate more Rap1 activation and B-cell spreading (Freeman et al., 2011). Cx43 prolongs Rap1 activation and enhances B-cell spreading through a mechanism involving its CT domain, (and more specifically, tyrosine residue Y265 may act as a phosphorylation site). The Cx43 CT domain may bind to adaptors of the cytoskeleton that i) stabilize cytoskeletal dynamics leading to cell spreading, ii) stabilize the formation of BCR micro-clusters or membrane micro-domains important for prolonged BCR-signaling, or iii) the CT domain may bind to effectors of the BCR signaling cascade, recruiting them to localized regions of the plasma membrane.
Adhesion proteins are typically transmembrane proteins that contain an extracellular domain capable of binding to extracellular matrix or cellular ligands, paired with a cytoplasmic domain that connects to the cytoskeleton directly or through binding to adaptor proteins. This link between the cytoskeleton and the extracellular substratum is important for adhesion, and contributes to dynamic processes like spreading and migration by generating tension that opposes the retrograde force generated by actin polymerization during the pushing forward of the leading edge of a migrating or spreading cell (Parsons et al., 2010).

We found the effects of Cx43 expression to be independent of GJ coupling in our system by using an *in vitro* assay that excluded cell-cell contact. This suggests that Cx43 does not function as an adhesion protein itself, however Cx43 may facilitate adhesion by linking the cytoskeleton to the plasma membrane at micro-domains where they associate with adhesion proteins that are able to connect the cell with the substratum (Figure 4.2 B i).

One caveat of this model is that it fails to explain how Cx43 expression prolongs Rap1 activation in response to BCR stimulation. BCR crosslinking induces a signaling cascade that leads to activation of the Rap1 GTPase. B-cell spreading increases the surface area of the cell that is in contact with Ag (or in this case anti-BCR) leading to increased BCR engagement and signaling leading to Rap1 activation. However, since Rap1 activation was measured in response to stimulation with soluble anti-BCR, cell spreading itself probably did not play a role in enhancing BCR signaling leading to the activation of Rap1.
A second way that scaffolding with the cytoskeleton could explain the effects of Cx43 expression, is if Cx43 enhances BCR-signaling through the stabilization of BCR micro-clusters or by altering the composition of plasma membrane micro-domains by regulating the proteins associated with the BCR. BCR signaling leads to the activation of the Rap1 GTPase (McLeod et al., 1998). Rap1 regulates the activity of proteins that modify actin dynamics, leading to cell-spreading (McLeod and Gold, 2001), as well as the actin-severing protein cofilin, which is necessary for the breakdown of the cortical actin cytoskeleton restricting the movement of BCRs within the plasma membrane (Freeman et al., 2011). Breakdown of the actin cytoskeleton frees up actin monomers for actin polymerization driving spreading. It also increases BCR clustering, which amplifies Rap1 activation through positive feedback (Freeman et al., 2011) (Figure 4.2 A). Cx43 could influence BCR-mediated cell spreading by regulating the activity of Rap1 if Cx43 could link to the cortical actin cytoskeleton underlying the plasma membrane and restrict the movement of the BCR or associated proteins. By enhancing BCR signaling, Cx43 could prolong the activation of Rap1, which regulates the activity of cytoskeletal binding proteins, leading to B-cell spreading (Figure 4.2 B ii).

BCR signaling is initiated by BCR crosslinking and the formation of BCR micro-clusters that act as the functional units of signaling (Harwood and Batista, 2009). Initial activation of Rap1 downstream of BCR signaling leads to actin severing that enhances mobility of BCR in the plasma membrane and facilitates the formation of BCR micro-clusters, enhancing BCR signaling and further Rap1 activation. The importance of the cytoskeleton on the regulation of BCR signaling can be inferred from the discovery that disruption of the actin cytoskeleton alone is sufficient to induce BCR clustering and signaling (Treanor et al., 2011).
Since there is no defect in initial Rap1 activation by B-cells lacking Cx43 expression (Machtaler et al., 2011), it seems that Cx43 is not required for initial BCR signaling itself, but rather for a sustained signaling response. By binding to the cytoskeleton through its CT domain, Cx43 may generate “picket fences” that restrict the mobility of proteins like the BCR in the plasma membrane (Kusumi and Sako, 1996). After BCR-mediated cell spreading, BCR microclusters aggregate into a well organized immune synapse arrangement with BCR in the center and adhesion proteins surrounding the periphery of this cluster (Figure 1.2). These events indicate that a massive reorganization of protein distribution in the plasma membrane takes place in response to BCR signaling, and one possibility is that Cx43 expression improves B-cell spreading by helping to sustain BCR signaling leading to Rap1 activation through the stabilization of signaling BCR clusters.

In addition to adhesion proteins that bind extracellular ligands directly, cell surface proteins can influence adhesion indirectly by contributing to micro-domains in the plasma membrane, thereby stabilizing the interactions of adhesion protein with the substratum. These plasma membrane domains can be restricted by the interactions of embedded proteins themselves or by their proximity to cytoskeletal-anchoring membrane proteins that restricting the movement of neighboring proteins by association. The tetraspanin family of transmembrane proteins, which share some similarities structurally with Cxs (four TM domains, a cytoplasmic NT and CT), have been shown to act as facilitators in processes such as cellular activation, proliferation, adhesion, and motility (Hemler, 2005), and to do so, not through ligand-binding or enzymatic activity, but through their interactions with other cell surface
proteins and/or through acting as scaffolding proteins via their cytoplasmic CT domains (Hemler, 2005).

Cx43 may be acting on BCR signaling or the spreading resulting from its stimulation, through a mechanism similar to the tetraspanin family: namely by influencing either micro-domain stability/composition, or by interactions with the cytoskeleton or other proteins through its CT domain. Many tetraspanins have been shown to co-localize with integrins and CD151 has been shown to bind integrins directly, in a mechanism involving the CT domain (Tejera et al., 2013). It has been suggested that CD151 influences cellular adhesion and motility by interactions with integrins that influence Rho GTPases, which are major regulators of actin dynamics in cytoskeletal reorganization. As another example, CD81’s importance in B-cell activation has been demonstrated by experiments in CD81−/− mice. It has been shown that CD81−/− results in less surface expression of CD19, a co-stimulatory molecule involved in BCR signaling (Shoham et al., 2003), as well as reduced recruitment of CD19 to BCR signaling micro-clusters in stimulated B-cells (Cherukuri et al., 2004).

Cx43 could act like a tetraspanin in this way: anchoring to the cytoskeleton and influencing BCR mobility or interaction with other components required for BCR signaling. Alternately, Cx43 could act as an adaptor to the cytoskeleton influencing actin dynamics directly through protein-interaction motifs located in its CT region.

While this model of Cx43 binding to cytoskeletal proteins or adaptors explains the influence that Cx43 has on Rap1 activation in response to BCR stimulation, work by MSc student Kate
Choi has shown that Cx43 knockdown specifically affects BCR signaling pathways leading to proximal events like cytoskeletal remodeling, but not pathways that lead to translocation to the nucleus of transcription factors that regulate the expression of genes involved in survival, growth, and proliferation (Guan et al., 1996). Considering the specificity of BCR signaling pathways affected, Cx43 may act downstream of BCR stimulation, rather than on BCR clustering itself.

Although Cx43 has been shown to bind directly to cytoskeletal adaptors, the CT domain contains sites for interactions with kinases and potentially for other adaptors as well, that may serve to recruit proteins to the plasma membrane at GJ plaques, or other Cx43-enriched membrane micro-domains. Cx43 could interact with proteins that are directly involved in the BCR signaling cascade by recruiting effectors to the plasma membrane or to specific plasma membrane domains. Localized recruitment of effectors could account for the effects of Cx43 knockdown and overexpression on cellular polarity (Francis et al., 2011a). As an example of recruitment, the BCR co-receptor CD19 lowers the threshold for signal transduction by recruiting Src family kinase Lyn and Syk to micro-domains containing the BCR (Depoil et al., 2007; Wang et al., 2012). Cx43 may also be able to bind Src family kinases involved in BCR signaling through its proline-rich domain or tyrosine residues, recruiting them to sites at the plasma membrane where they are better able to phosphorylate tyrosine residues of the BCR ITAMs upon BCR-stimulation (Figure 4.2 B iii).

Understanding the role of Cx43 in B-cell spreading and on cytoskeletal rearrangement in general have implications for understanding B-cell normal function and during an immune
response. Cytoskeletal dynamics control B-cell morphology and motility, which are required for B-cell interactions with their environment throughout development, immune surveillance, and the generation of an immune response. In particular, the process of B-cell spreading increases the formation of BCR-micro-clusters, leading to immune synapse formation and B-cell activation by membrane-bound Ag (Carrasco et al., 2004; Fleire et al., 2006). In our system, B-cells were stimulated by treatment with either soluble anti-IgM (Chapter 2.3.3) or by plating on glass coated with anti-IgM (Chapter 2.6.1), which served to crosslink the BCR and result in BCR signaling and B-cell spreading. Although B-cell spreading was initiated by anti-IgM coated glass, B-cells most commonly recognize membrane-bound Ag on an APC that may contain many other adhesion and co-stimulatory molecules on its surface. To more closely model B-cell activation, the effect of Cx43 on BCR-mediated cell spreading could be tested in B-cells cultured on an artificial lipid bilayer loaded with Ag and other proteins involved in B-cell spreading and activation. To look at later events in B-cell activation such as immune synapse formation and B-cell activation, B-cells could be co-cultured with either surrogate APCs that have been loaded with Ag or with actual APCs such as primary dendritic cells.

In vivo studies of the role of Cx43 in the adaptive immune system have been limited to the importance of Cx43 for lymphocyte development (Montecino-Rodriguez et al., 2000), since Cx43−/− mice are perinatal lethal and Cx43+/− mice have few phenotypes. A possible strategy to investigate the importance of Cx43 in the immune response of adult mice would be to adoptively transfer bone marrow from Cx43−/− mice into an irradiated Cx43+/+ host (Nguyen and Taffet, 2009). A more specific approach would be to make B-cell specific conditional
knockout mice by crossing Cx43-floxed mice (Liao et al., 2001) with mb-1/Cre mice (Hobeika et al., 2006).

In summary, this project explored three possible functional domains of Cx43: The extracellular loops, the channel, and the cytoplasmic CT tail. The six cysteine residues in the extracellular loops of Cx43 were shown to be important for trafficking of Cx43 to the plasma membrane since mutations of two or more cysteine residues resulted in a larger intracellular pool of Cx43. B-cells did not form HCs except upon overexpression of Cx43, which may overload the ability of endogenous cellular machinery to regulate HC conductance. The influence of Cx43 on BCR-mediated B-cell spreading was shown to be channel-independent by treatment with channel blocking agents, which has no effect on spreading, even in Cx43-overespressing cells with measurable HC activity.

The well-studied Cx43 channel mutant Cx43T154A prevented normal B-cell spreading in a dominant negative manor, but we suspect that it may do so because of a conformational change to the cytoplasmic CT tail or due, at least, to a combined role of the tail in channel gating since Cx43 mutants Cx43T154A and Cx43Δ246 both abrogate BCR-mediated Rap1 activation, and since the added insult of a Cx43Δ246 truncation to the mutant Cx43T154A prevents an intermediate non-radial spreading phenotype that may otherwise have been attributed to channel conductance alone.

In further investigating the importance of the Cx43 tail, we found that mutation of a single tyrosine residue of the cytoplasmic tail Y265 is sufficient to block spreading, and hypothesize
that this site is bound by a Src kinase family member downstream of BCR signaling, following this residue's phosphorylation. Cx43’s influence on B-cell spreading seems linked to activation of the master regulator of the B-cell cytoskeleton: Rap1 GTPase. However, it remains to be determined whether Src kinases play a role in Cx43 effect on Rap1 activation, and whether Cx43 participates in signaling cascades or anchors the cytoskeleton through adaptor proteins.
REFERENCES


APPENDIX

A1 Cx43 extracellular cysteine residues are required for proper trafficking to the plasma membrane

Initially, the purpose of this project was to study the role of the Cx43 extracellular loops on B-cell spreading. Conflicting reports suggested that either the Cx43 cytoplasmic tail (Bates et al., 2007) or extracellular loops (Elias et al., 2007) were responsible for neuronal migration since mutations of these domains were shown to prevent GOF in their respective study. Both studies agreed that Cx43’s importance for neuronal migration was independent of the channel since channel-blocking drug CBX (Bates et al., 2007) and channel-blocking mutant Cx43T154A (Elias et al., 2007) did not impair migration. Previous work had shown that the CT domain was required for B-cell spreading (Machtaler et al., 2011) and so we wondered if the extracellular domains would be required since both spreading and migration depend upon cytoskeletal rearrangement and anchorage to a substrate through adhesion. Each extracellular loop of Cx43 contains three cysteine residues that form disulphide bonds with adjacent connexons, connecting neighboring cells via GJs. Elias et al. hypothesized that GJs formed between cells could provide traction for migration. We expected that the CT and the extracellular domains might be important if they worked together to transmit tension from the extracellular environment via the disulphide bonds to the cytoskeleton via the Cx43 cytoplasmic tail.
**Table A1. Cys-less (CL) mutants generated by site directed mutagenesis.** Data is summarized from three independent experiments with RV-transduced J558µm3 cells expressing CL Cx43-EGFP. Localization was taken from cells immobilized on poly-L-lysine coated coverslips using confocal microscopy. Green=WT or mutant Cx43-EGFP. Scale bars represent 10 µm. Molecular weight was estimated from western blot verification of expression (Figure A1).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Mutation</th>
<th>Found at the Plasma Membrane?</th>
<th>Localization</th>
<th>Molecular Weight</th>
</tr>
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<tr>
<td>EGFP</td>
<td></td>
<td>-</td>
<td></td>
<td>27 kDa</td>
</tr>
<tr>
<td>Cx43</td>
<td></td>
<td>++ +</td>
<td></td>
<td>72 kDa</td>
</tr>
<tr>
<td>CL2</td>
<td>C61-65A</td>
<td>+</td>
<td></td>
<td>45-80 kDa (smear)</td>
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<tr>
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<td>-</td>
<td></td>
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<tr>
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<td>C54-61-65-192-198A</td>
<td>+</td>
<td></td>
<td>72 kDa</td>
</tr>
<tr>
<td>CL6</td>
<td>C54-61-65-187-192-198A</td>
<td>-</td>
<td></td>
<td>72 kDa</td>
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</table>
Figure A1. Expression of CL mutants in a B-cell line. Western blot showing expression of “cys-less” (CL) Cl2, Cl3, CL5 and CL6 in the J558μm3 cell line stably transduced by retroviral infection (Blot was stripped and re-probed for actin as a loading control). Data is representative of three independent replicates.
Site Directed Mutagenesis was performed in successive rounds on EGFP-fused Cx43 in the NAP2 plasmid (Bates et al., 2007). Mutants were named for the number of cysteine to alanine substitutions they contained in order from the N to the C terminus: “Cys-less” (CL): Cx43CL2, Cx43CL3, Cx43CL5, and Cx43CL6 (Table A1). All four mutants were expressed in the J558µm3 cell line by retroviral transduction (Chapter 2.3.2). Cells were periodically re.sorted for EGFP to maintain expression (Chapter 2.5). Expression was confirmed by western blot (Figure A1), however immunofluorescence showed that CL Cx43 was mostly retained intracellularly (Table A1). Trafficking defects have been noted for cysteine mutations of Cx43 in the literature (Tong et al., 2007) and since our In vitro assays depend heavily on good surface expression of Cx43, the decision was made, with the help of my committee, to abandon this portion of the project in favor of the channel and tail domains which had been unavailable to study when I started (these domains were under investigation by previous lab members).
A2 Mutation of Cx43 extracellular cysteine residues prevents B-cell aggregation

Based on the observation that the expression of different Cx43 mutants affected the clumpiness of B-cell lines in tissue, an assay was developed to measure the aggregation of B-cells in suspension (Chapter 2.4.6). B-cells were cultured at low densities in low serum media to prevent cell division over a 48 h time-course. Images of cultured cells were collected and then all objects per field were quantified and their areas divided by the area of a single cell, to estimate the number of cells/clump (Figure 2.2).
Figure A2. Cx43 expression leads to J558µm3 cell aggregation and is dependent on Cx43 extracellular cysteine residues. A) Images of J558µm3 cells in suspension after dispersing into single cells (0 h), and after aggregation (48 h). Wehi231 cells were used as a positive control for aggregation, and un-transfected J558µm3 as a negative control. B) Quantification of cell aggregation by #cells/aggregate (see Chapter 2.6.4). Error bars represent standard error of the mean. Data is representative of three independent experiments, in which 10 fields of cells were quantified for each sample.
Samples were pipetted up and down at the start of the experiment to dissociate any aggregates into dispersed single cells. Although J558µm3 cells do not normally aggregate in tissue culture, by 5hs J558µm3 cells expressing WT Cx43 and Cx43T154A had started to form small clumps of 2-3 cells. By 24 hs, aggregates of as many as 20 cells had been formed by the cells expressing Cx43T154A, which had diverged significantly from the WT Cx43 expressing cells which formed smaller aggregates of about 15 cells. By 48 hrs, EGFP-expressing or Cx43CL6-expressing cells still had not aggregated at all, whereas WT Cx43 expression induced B-cell aggregation, resulting in the formation of ~20 cell aggregates, and expression of closed-channel mutant Cx43T154A increased aggregation significantly as compared to WT Cx43 by forming aggregates of as many as 80 cells (Figure A2).

Cx43 expression has been reported to influence the aggregation of other cell types. For example, overexpression of Cx43 in C6 glioma cells increases their aggregation in vitro (Lin et al., 2002). Expression of Cx43 cysteine mutant Cx43C61S was not sufficient to cause C6 aggregation, however expression of a closed-channel Cx40/43 chimera increased aggregation (Lin et al., 2002), consistent with our findings regarding the expression of cysteine-less Cx43CL6 and closed-channel mutant Cx43T154A respectively.

Aggregation is predictive of the adhesivity and invasion of C6 glioma in vivo. While J558µm3 cells do not normally aggregate in vitro, blood cell homotypic aggregation is diagnostic of myelomas (Kawano et al., 2008) and could demonstrate effects of Cx43 expression on B-cell adhesivity. It is unclear from this data, whether Cx43 expression results in aggregation due to GJ formation between B-cells or due to altered expression of some other adhesion protein on
the cell surface. Knockdown of Cx43 has no effect on integrin LFA-1 surface expression as measured by FACS (*Kate Choi, data not shown*), however loss of Cx43 and E-cadherin expression are correlated in many cancer tissues and cancer cells lines (Li et al., 2008; Xu et al., 2008) and GJIC may influence the expression of E-cadherin (Yu et al., 2012), suggesting that Cx43 could influence aggregation through non-Cx adhesion proteins as well.