CHARACTERIZATION OF THE EFFECTS OF CONNEXIN43 ON B CELL RECEPTOR SIGNALING RESPONSES IN B-LYMPHOCYTES

by

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Abstract

Reorganization of the cytoskeleton is crucial in orchestrating B lymphocyte development and immune responses. Our lab aims to understand the mechanisms of B cell cytoskeleton regulation. Intracellular signaling from the B cell receptor (BCR) triggers cytoskeletal changes necessary for B cell activation. Connexin43 (Cx43) is a gap junction-forming protein that, when expressed in J558µm3 Cx43-negative B cells, is sufficient to enable BCR-mediated cellular spreading, a process in which cytoskeleton remodeling drives changes in cell shape. Knockdown of Cx43 in WEHI231 B cells reduced the activation of various BCR signaling proteins, including the Rap1 GTPase, a master regulator of cytoskeleton dynamics in B cells. Since the carboxyl tail (CT) of Cx43 was important for this increased Rap1 activation, we investigated the role of the Cx43 CT in B cell cytoskeleton regulation by characterizing its effects on BCR signaling responses. Our approach assessed J558µm3 cells transfected with various Cx43 CT mutant constructs to determine the role of Cx43 in BCR signaling. We found that Rap1 can be activated by mechanical forces applied to cells, and that physical expansion of cell size by stretching in the absence of receptor stimulation caused no apparent changes to the actin cytoskeleton. Because Cx43 had no impact on these results, it likely has the most influence in BCR signaling pathway-driven cytoskeletal changes. The Cx43 CT, in particular serines 279 and 282, but not 255 and 262, were found to be important for BCR-induced tyrosine phosphorylation of cellular proteins (pTyr), indicating a differential role of specific amino acids in the Cx43 CT on BCR signaling. The phosphorylation of Ezrin / Radixin / Moesin (ERM) proteins was also decreased upon the loss of Cx43 and its CT. We speculate that the CT recruits protein partners to act with BCR signaling enzymes. Cx43 may also control cytoskeleton dynamics via regulating ERM proteins. Future studies include monitoring pTyr in additional Cx43 CT mutants to identify other residues important for BCR signaling and uncovering how Cx43 contributes to ERM phosphorylation. This work provides insight into cytoskeletal rearrangement in B cells, a process in which dysregulation underlies B cell autoimmune diseases and cancers.
Lay Summary

A cell’s cytoskeleton is a dynamic structure that provides mechanical support to the cell and can be easily rearranged. Our immune system is made up in part by white blood cells known as B cells, and their job is critically dependent on them being able to remodel their cytoskeleton. One of the ways B cells can do this is by receiving information from proteins on their surface that detect signals derived from invading germs. We found that another protein named Connexin43 may be a key player in this process because removal of Connexin43 impairs the ability of B cells to reorganize their cytoskeleton. Further studies also indicated that a loss of Connexin43 results in decreased B cell signal transmission and activation of cytoskeleton-associated proteins. These findings could help understand cytoskeleton remodeling in B cells and may contribute to new treatments for B cell diseases that involve dysregulation of the cytoskeleton.
Preface

The research done in this thesis was developed collaboratively by Dr. Linda Matsuuchi and Victor Cheuk-Kong Mo, with valuable input from Dr. Calvin Roskelley and Dr. Christian Naus.

May Dang-Lawson was responsible for preparing cell lysates in three of the six replicates done for Figure 3.10 A and B. The representative blots shown in Figure 3.10 A and B are from experiments done by May Dang-Lawson. All remaining experiments were carried out by Victor Cheuk-Kong Mo.
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List of Abbreviations

- Minus
# Number
+ Positive
°C degrees Celsius
2-ME β-mercaptoethanol
7-AAD 7-Aminoactinomycin D
α- Anti
α Alpha
β Beta
Δ Truncation
µg Microgram
µl Microliter
μm Micrometer
µM Micromolar
A Alanine
aa Amino acid
ABL Abelson family non-receptor tyrosine kinases
AID Activation-Induced Deaminase
Akt Protein kinase B
APC Antigen-presenting cell
Arp2/3 Actin related protein 2/actin related protein 3
ATCC American type culture collection
ATP Adenosine triphosphate
BAR Bin / amphiphysin / rvs
BCA Bicinchoninic acid
BCAP B-cell adaptor for PI3K
BCR B cell receptor
BSA Bovine serum albumin
BLNK B cell linker protein
Btk Bruton’s tyrosine kinase
CaCl2 Calcium Chloride
CalDAG-GEF Calcium- and Diacylglycerol-regulated guanine exchange factor
CD Cluster of differentiation
Cdc42 Cell division control protein 42
cDNA Complementary deoxyribonucleic acid
CK1 Casein kinase 1
CMV Cytomegalovirus
CO2 Carbon dioxide
Co-IP Co-immunoprecipitation
cSMAC Central supramolecular activation cluster
CT Carboxyl tail
Cx Connexin
Cx43 Connexin43
DAG Diacylglycerol
DC Dendritic cell
DMSO Dimethyl sulfoxide
DNP Dinitrophenyl
DTT 1,4-Dithiothreitol
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin / Radixin / Moesin</td>
</tr>
<tr>
<td>F</td>
<td>Filamentous</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FceRI</td>
<td>Fc epsilon receptor 1</td>
</tr>
<tr>
<td>FER</td>
<td>Fps/Fes related tyrosine kinase</td>
</tr>
<tr>
<td>FERM</td>
<td>Four point one Ezrin Radixin Moesin</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G</td>
<td>Gauge</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GRP</td>
<td>Guanyl releasing protein</td>
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<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H + L</td>
<td>Heavy and light chains</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HI-FBS</td>
<td>Heat-inactivated fetal bovine serum</td>
</tr>
<tr>
<td>HS1</td>
<td>Cortactin-like protein haematopoietic lineage cell-specific protein 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular Adhesion Molecule 1</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IgA</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IGEPAL</td>
<td>Octylphenoxy polyethyleneoxyethanol</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IP3</td>
<td>Inositol-1,4,5-trisphosphate</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
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<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KC1</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LOK</td>
<td>Lymphocyte oriented kinase</td>
</tr>
<tr>
<td>LSI</td>
<td>Life sciences institute</td>
</tr>
<tr>
<td>Lyn</td>
<td>Lck/Yes novel tyrosine kinase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>Mek</td>
<td>MAPK/ERK kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>M.Sc.</td>
<td>Master of science</td>
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<tr>
<td>MTOC</td>
<td>Microtubule-organizing center</td>
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<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>Sodium hydroxide</td>
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<td>Na₂HPO₄</td>
<td>Sodium hydrogen phosphate</td>
</tr>
<tr>
<td>Na₃VO₄</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>NEDD4</td>
<td>Neural precursor cell-expressed developmentally downregulated gene 4</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor of κ-binding</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PLCγ2</td>
<td>Phospholipase Cγ2</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Protein tyrosine kinase 2</td>
</tr>
<tr>
<td>RalGDS</td>
<td>Ral guanine nucleotide dissociation stimulator</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
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<td>RAPL</td>
<td>Rap ligand</td>
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<td>RIAM</td>
<td>Rap1-GTP-interacting adapter molecule</td>
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<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<td>Serine</td>
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<td>Sodium dodecyl sulfate</td>
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<td>Src family kinase</td>
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<td>Src Homology 2</td>
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<tr>
<td>SHP-2</td>
<td>Src-homology-2-domain-containing protein tyrosine phosphatase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>SLK</td>
<td>STE20-like kinase</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TC-PTP</td>
<td>T cell protein tyrosine phosphatase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Tyk2</td>
<td>Tyrosine Kinase 2</td>
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<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>UBC</td>
<td>University of British Columbia</td>
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<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen 4</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP-family verprolin-homologous protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
</tr>
</tbody>
</table>
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Chapter 1: Introduction

1.1 The immune system

Our immune system defends the body against infection by pathogens such as bacteria and viruses. Vertebrate immunity consists of two main modes. The innate immune system is characterized by non-specific immunity against a broad range of pathogens and is immediately available upon encounter with infectious agents. This response does not lead to lasting immunity, which is one of the main differences that set it apart from the adaptive immune system. Adaptive immunity is developed slowly in the early stages of infection but confers lifelong immunological memory that allows the body to respond more rapidly if re-infected by the same pathogen (Lampropoulou et al., 2012).

The innate immune system is one of the first lines of defense against infection and includes dendritic cells (DC), macrophages, mast cells, and neutrophils (Chaplin, 2003). These cells express germline-encoded pattern-recognition receptors (PRRs) such as the Toll-like receptors (TLRs) that detect conserved pathogen-associated molecular patterns (PAMPs) like bacterial lipopolysaccharides and viral nucleic acids (Iwasaki and Medzhitov, 2015). Binding of PAMPS to innate cells lead to enhanced phagocytosis and degranulation abilities, as well as induction of neutrophil extracellular traps for improved trapping and killing of invading microorganisms (Iwasaki and Medzhitov, 2015; Brinkmann et al., 2004). Innate immune cells specialize in preventing immediate pathogenic spread, and their main purpose is to resolve infection without resorting to adaptive immunity (Janeway and Medzhitov, 2002).

An adaptive immune response is required when innate defenses have been evaded or overwhelmed. It is the strongest form of immunological defense headed by T and B cell lymphocytes. While innate cells provide immediate protection, adaptive immunity needs time to develop. Generation of T and B cells involve sequential recombination of T cell receptor and B cell receptor
(BCR) genes, as opposed to germline-encoded PRRs. Each receptor combination is only expressed by a single cell but populations of T and B cells as a whole express a virtually unlimited number of receptors able to recognize a vast array of pathogenic antigens (Lampropoulou et al., 2012). Dendritic cells can instruct T cells on the nature of the invaders by presenting processed antigens to elicit T cell activation (Steinman, 2001). Activated T cells directly kill infected cells or produce cytokines to recruit phagocytic macrophages and activate B cells to promote antibody-mediated immunity. Activation of B cells is initiated by engagement of their BCR with intact antigen, followed by interactions with CD4+ helper T cells to complete B cell differentiation into plasma cells and long-lived memory cells (more in section 1.2.1). Plasma cells produce highly-specific antibodies to neutralize pathogens or tag them for complement activation or enhanced phagocytosis by macrophages. If the pathogen is encountered again in future infections, they are immediately recognized by memory B cells; there is no delay in the production of antibodies. Immunological memory continually refines an individual’s antibody arsenal such that antibodies become increasingly specific and bind with greater affinity to its target. Thus, a stronger immune response is mounted with each invasion by the same pathogen (Lampropoulou et al., 2012; Selvaraj et al., 2016; Janeway and Medzhitov, 2002), a major advantage of the adaptive immune system.

1.2 B Lymphocytes

1.2.1 Humoral immunity

The main duty of B cells in the adaptive immune response is to provide humoral (e.g. antibody-mediated) immunity. To do this, they must first become activated by antigen. Mature B cells constantly circulate the body surveying for antigens that are recognizable by their BCR. As they migrate around the body, they enter secondary lymphoid organs, such as the spleen, where antigen is concentrated. These environments increase the chances of B cell and antigen encounter as well as interactions with T cells. Here, B cells can come across intact antigen displayed on follicular dendritic cells and subcapsular sinus macrophages or small soluble antigens that may have been released from
proteolysis of larger antigens (Batista and Harwood, 2009; Cyster, 2010). Clustering of BCRs by antigen triggers immune synapese formation to facilitate BCR-antigen internalization into lysosomal vesicles where the antigen is processed into peptide fragments and recycled back onto the surface of the B cells bound to major histocompatibility class II complexes (MHC II) (Selvaraj et al., 2016) (see more in section 1.3.3). However, antigenic stimulation alone results in only transient B cell activation that eventually progresses to cell death or anergy, a state of immune unresponsiveness, because pro-apoptotic signals are also initiated (Rawlings et al., 2017). Complete activation is only successful if the B cell also receives costimulatory signals from T cells or its TLRs to ensure the body only mounts an antibody response if actual indicators of microbial infection (e.g. bacterial lipoproteins) are present (Palm and Medzhitov, 2009). Activated CD4+ helper T cells deliver the necessary costimulatory signal to B cells via their surface CD40 ligand (CD154) expression and IL-4 cytokine secretion. Continued CD40 ligand expression and IL-4 secretion require reciprocal stimulation by the activated B cell in the form of peptide-MHC II complexes and CD80/CD86 expression on the B cell surface, which bind to the T cell receptor and CD28, respectively. In this manner, the B cell acts as an antigen-presenting cell (APC) critical for full T cell activation (Vascotto et al., 2007; Yuseff et al., 2011). Antigenic stimulation via the BCR together with costimulatory signals provided by T helper cells or TLR engagement in combination drive B cell activation, proliferation, and differentiation into antibody-producing plasma cells and memory B cells (Rawlings et al., 2017; Abraham et al., 2016), thus establishing the basis of humoral immunity.

1.2.2 The B cell receptor

1.2.2.1 Structure

The B cell receptor is a transmembrane signaling complex that controls B cell fate from its development to its activation process to become terminally-differentiated plasma cells. It consists of an antigen-recognition subunit paired to a signal transduction subunit. The ligand-binding subunit of the BCR is an immunoglobulin (Ig) molecule that is the transmembrane form of secreted antibodies.
Each membrane Ig consists of two identical heavy (H) and light (L) chains joined together by disulfide bonds. The amino-terminus of these chains contain highly variable (V) regions which combine ($V_H$ and $V_L$ together) to create two identical antigen-binding sites for each membrane Ig protein (Reth and Nielsen, 2014). These binding sites have enormous diversity as a result of genetic rearrangements on light chain variable regions early on during B cell development (Zhong et al., 2014). Successful L chain gene rearrangements can only occur at either the loci encoding the κ or λ light chains and as such each B cell and its progeny will only produce one type of L chain. The ratio of κ and λ light chain usage in mice is approximately 95% κ and 5% λ but no functional differences between B cell receptors that contain either of these L chains have been found (Melchers, 2015). At later stages of B cell maturation, variable regions are further modified by activation-induced deaminases (AID) through a process termed somatic hypermutation in which B cell receptor genes undergo extremely high rates of mutations as B cells proliferate. Ultimately numerous B cells are produced with slightly differing receptors and varying antigen specificity such that B cells expressing BCRs with the greatest affinity for its antigen are selected to continue development (Durandy, 2003).

Immunoglobulin can be of all five Ig isotypes (IgM, IgD, IgG, IgA, and IgE) depending on the class of their heavy chain (µ, δ, γ, α, ε, respectively). Naive mature B cells express both IgM and IgD but will undergo class switch recombination after activation by antigen. Class switching does not alter the variable region of the heavy chain and therefore ligand binding is not changed. Rather, the secreted antibody retains its antigen specificity and can interact with different effectors to elicit different immune functions (e.g. IgE binding to Fc receptors induces mast cell degranulation) (Venkitaraman et al., 1991). Together, the antigen-recognition subunit consists of two identical variable ligand-binding regions connected to constant regions of the Ig heavy chains. Surface Ig does not possess enzymatic activity, and is instead non-covalently associated with the Igα/Igβ (CD79α/β) heterodimeric signal transduction subunit. Both CD79α and CD79β contain cytoplasmic immunoreceptor tyrosine based activation motifs (ITAM). Phosphorylation of conserved tyrosine residues on these ITAMs recruit tyrosine kinases to initiate downstream BCR signaling pathways (Abraham et al., 2016). In this way,
antigen binding by the immunoglobulin subunit translates into a biochemical signal through phosphorylation of the signal transduction subunit.

1.2.2.2 B cell receptor signaling

Signaling from the B cell receptor serves two main functions: it provides pro-survival differentiation signals to B cell progenitors and drives the activation of mature B cells to become antigen-presenting cells that are capable of eliciting further differentiation signals from CD4+ helper T cells (Zhong et al., 2014). In the latter case, antigen-induced BCR aggregation leads to tyrosine phosphorylation of ITAMs by Src family kinases (SFK) such as Lyn, Fyn, and Blk (Stepanek et al., 2013). Lck/Yes novel tyrosine kinase (Lyn) is highly expressed in B cells and has been shown to be the predominant initiator of the BCR-derived signaling cascade (Yamanashi et al., 1991; Campbell and Sefton, 1992). Lyn also associates with CD19, a transmembrane protein that can act as a coreceptor for the BCR. Therefore, BCR-CD19 clustering can also lead to phosphorylation of BCR ITAMs (Abraham et al., 2016). Phosphorylated tyrosine residues within ITAM motifs create docking sites for SH2-domain-containing proteins such as the spleen tyrosine kinase (Syk). Tyrosine phosphorylation of Syk by nearby SFKs activates it and allows it to phosphorylate the B-cell adaptor for PI3K (BCAP) as well as CD19 to create binding sites for recruitment of Phosphoinositide 3-kinase (PI3K) (Okada et al., 2000). Syk also phosphorylates the B cell linker protein (BLNK) to serve as an assembly focus to recruit key BCR signaling proteins including phospholipase Cγ2 (PLCγ2), Bruton’s tyrosine kinase (Btk), Vav, and the adaptor growth factor receptor-bound protein 2 (Grb2) that contribute to B cell activation (Jumaa et al., 2005; Zhong et al., 2014). Many of these proteins also become tyrosine phosphorylated by Lyn or Syk (Patterson et al., 2006). Together they form the BCR “microsignalosome” complex. At this point the cascade is split into the PI3K and downstream Ca²⁺ pathways, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, and the nuclear factor of κ-binding (NF-κB) pathway (Hobeika et al., 2015) that together promote B cell activation, survival, and proliferation.
The PI3K pathway begins with conversion of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 is responsible for recruiting proteins containing pleckstrin homology (PH) domains such as protein kinase B (Akt), Btk, and PLCγ2 to the plasma membrane where they can anchor themselves to phospholipids (Lemmon and Ferguson, 2000) and facilitate their activation (Hobeika et al., 2015). Akt promotes B cell survival and proliferation in part through activating the mechanistic target of rapamycin (mTOR) complex, which controls protein synthesis (Blachly and Baiocchi, 2014). Btk is mainly responsible for activating PLCγ2 (Kurosaki et al., 2000), which is recruited to the plasma membrane by its association with BLNK. Activated PLCγ2 converts PIP2 into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG activates Protein kinase C (PKC), which causes nuclear translocation of NF-κB (Cantrell, 2015) while IP3 triggers an increase in intracellular calcium (Hendriks et al., 2014). DAG is also important for the activation of Rap1 GTPases (Abraham et al., 2016), an important regulator of cytoskeleton organization in B cells (McLeod et al., 1998; Lin et al., 2010) (see more in section 1.3.4). DAG and calcium ions regulate the activation of guanine nucleotide exchange factors (GEF) such as Ras guanyl releasing protein (RasGRP) to cause an increase in Ras-GTP levels (Oh-hora et al., 2003). Ras-GTP propagates the MAPK Ras-Raf-Mek-ERK cascade leading to enhanced B cell proliferation and survival (Genot and Cantrell, 2000). IP3-induced calcium increase also leads to activation of the NFAT transcription factor complex which, together with NF-κB cause changes to gene expression, cytokine production, and contributes to B cell survival and eventual differentiation with T cell help (Oh-hora and Rao, 2008; Hobeika et al., 2015) (Figure 1.1).

Negative regulators of BCR signaling also exist. Interestingly, Lyn-deficient mice have enhanced BCR signaling (Hibbs et al., 1995), which is now explained by that fact that Lyn has a dual function of facilitating activation of phosphatases to limit BCR signaling as well (Seda and Mraz, 2015). It does so by phosphorylating immunoreceptor tyrosine-based motifs (ITIMs) on CD22 and
Fcγ-receptor IIB to create docking sites for phosphatase and tensin homolog (PTEN) and Src-homology-2-domain containing inositol 5′ polyphosphatase (SHIP-1/2). These phosphatases remove the phosphate group from PIP3 thus reverting the action of PI3K in response to BCR signaling (Mayo and Donner, 2002; Brauweiler et al., 2000). Src-homology-2-domain-containing protein tyrosine phosphatase 1 (SHP-1) can also be recruited by phosphorylated ITIM motifs on CD22 and Fcγ-receptor IIB to dephosphorylate key proteins of BCR signaling including CD79α/β, Syk, BLNK, and Vav (Pao et al., 2007), thereby shutting down the signaling cascade.
Figure 1.1. The B cell receptor signaling pathway.
Major aspects of the B cell receptor signaling pathway. Binding of antigen to the BCR triggers formation of the BCR microsignalosome (signaling microcluster) after Lyn phosphorylates the ITAM motifs on CD79α/β. A BCR-CD19 interaction is important in recruitment of PI3K. PI3K catalyzes the conversion of PIP3 into PIP2. Akt is brought close to the plasma membrane by PIP3 where it can be activated. Akt then promotes protein synthesis via the mTOR complex. PLCγ2 converts PIP2 into IP3 and DAG. Together these mobilize intracellular calcium stores and activate the MAPK cascade to promote B cell proliferation and survival. DAG also activates the Rap1 GTPases, a key regulator of actin cytoskeleton remodeling. Y: Tyrosine; EC: Extracellular; IC: Intracellular; ITAM: Immunoreceptor tyrosine based activation motif; Lyn: Lck/Yes novel tyrosine kinase; Syk: Spleen tyrosine kinase; Btk: Bruton’s tyrosine kinase; Grb2: Growth factor receptor-bound protein 2; PLCγ2: Phospholipase Cγ2; BLNK: B cell linker protein; PI3K: Phosphoinositide 3-kinase; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; PIP2: Phosphatidylinositol 4,5-bisphosphate; Akt: Protein kinase B; mTOR: Mechanistic target of rapamycin; IP3: Inositol-1,4,5-trisphosphate; DAG: Diacylglycerol; Ca2+: Calcium ions; NFAT: Nuclear factor of activated T-cells; NF-κB: Nuclear factor of κ-binding; PKC: Protein Kinase C; ERK: Extracellular signal-regulated kinase. This figure is made with help from Abraham et al. Encyclopedia of Immunobiology. 2016, Hobeika et al. J Mol Med. 2015, and Zhong et al. Semin Hematol. 2014.
1.3 Importance of the cellular cytoskeleton in B cell immunity

1.3.1 The peripheral actin cytoskeleton

The cellular cytoskeleton provides mechanical support and a means of intracellular transportation for the cell. It is composed of a dense network of thread-like actin filaments (3-6 nm diameter) found mostly at the periphery of the cell and surrounds the intermediate filaments and microtubules (20-25 nm diameter) that are dispersed within the cell (Blanchoin et al., 2014). Many aspects of B cell immunity including BCR signaling, B cell spreading, and immune synapse formation is reliant on rearrangement of the cytoskeleton. As such, another element of BCR signaling is to induce reorganization of the cellular cytoskeleton (Kuokkanen et al., 2015). Actin has particular relevance in B cell receptor signaling because rearrangement of the actin cytoskeleton affects plasma membrane organization and thus receptor organization and signaling (Tolar, 2017). The peripheral actin cytoskeleton is generated by the activity of both the actin related protein 2/actin related protein 3 (Arp2/3) complex and formins. Arp2/3 is an actin polymerization nucleating factor that mediates branched F-actin formation on existing actin filaments while the formin family of actin nucleators cause de novo polymerization of straight filaments (Bovellan et al., 2014; Fritzsche et al., 2016). Together they form a meshwork of short actin filaments situated under the plasma membrane. Immunodeficiencies can be caused by defects in actin polymerization (Moulding et al., 2013). The most well-known disease of this type is Wiskott–Aldrich syndrome. This disease is caused by mutations to the Wiskott-Aldrich syndrome protein (WASP) (Becker-Herman et al., 2011) which renders it unable to activate the Arp2/3 complex (Derry et al., 1994; Thrasher and Burns, 2010). WASP-deficient B cells have increased BCR signalling and antibody production (Liu et al., 2013) and a majority of patients with Wiskott–Aldrich syndrome experience B cell hyperactivity that leads to antibody-mediated autoimmunity (Becker-Herman et al., 2011; Recher et al., 2012). This suggested that B cell activation is closely linked with cellular cytoskeleton organization.
1.3.2 Receptor mobility and BCR signaling

The actin meshwork attaches to the plasma membrane via the Ezrin / Radixin / Moesin (ERM) proteins and class I myosin actin-crosslinking proteins (Parameswaran and Gupta, 2013; Maravillas-Montero et al., 2011). The absence of additional phospho-tyrosine containing sequences in the CD79α/β signaling subunit suggests that the BCR cannot recruit other SH2-domain containing proteins to the BCR after Syk binding. However, tyrosine kinases that are already associated with the BCR can phosphorylate tyrosines on the CD19 coreceptor to create docking sites for other downstream BCR signaling effectors (Kelly and Chan, 2000; Myung et al., 2000). This highlights the importance of BCR-CD19 interactions in BCR signaling (Rickert et al., 1995). The mobility (and thus chances of interaction) of transmembrane proteins such as the BCR and CD19 are limited by the underlying actin cytoskeleton (Treanor et al., 2010). Actin filaments act as interlinking “fences” connected to transmembrane protein “pickets” via actin-linkage proteins such as ERM to segregate and limit the free diffusion of membrane proteins (Kusumi et al., 2005; Klammt and Lillemoier, 2012). Importantly, disruption of the actin network alone in the absence of antigen stimulation increases receptor diffusion and triggers B cell activation that is dependent on the BCR and CD19 (Treanor et al., 2010; Mattila et al., 2013). These data suggest that by restricting the diffusion of the BCR and its co-receptor the actin cytoskeleton is able to establish blockades to limit B cell activation (Kuokkanen et al., 2015). Notably, ERM proteins play an important role in BCR signaling by regulating receptor diffusion. Antigen stimulation leads to transient de-phosphorylation of Ezrin, which temporarily dismantles actin-mediated diffusion barriers and increases BCR mobility to promote BCR clustering (thus signaling) (Gupta et al., 2006). After formation of BCR microsignalosomes, Ezrin is re-phosphorylated and actin barriers are re-established around these BCR signaling clusters. Corralling by the actin meshwork has a net effect of increasing the chances of interactions between proteins within the area (Kalay et al., 2012). Therefore, reinstatement of actin barriers via ERM proteins maintain the integrity of BCR microsignalosomes and promote the
stabilization of BCR signaling (Treanor et al., 2011; Treanor et al., 2010) (discussed more in section 4.2.2).

1.3.3 B cell spreading and Immune Synapse formation

Antigen displayed on follicular dendritic cells can be recognized by cognate BCRs on B cells. As this happens, Ezrin is de-phosphorylated (Gupta et al., 2006), and the actin network is locally depolymerized by the Cofilin actin-severing protein (Freeman et al., 2011). This is followed shortly by the generation of large numbers of new actin filaments protruding outwards from the initial point of antigen contact to drive membrane expansion and cell spreading. B cell spreading maximizes the contact area between the B cell and the APC thereby promoting antigen encounter and coalescence of enough BCR signal microsignalosomes to form an immune synapse for antigen internalization (Fleire et al., 2006). Meanwhile, the microtubule-organizing center (MTOC) moves towards the site of immune synapse formation in response to BCR signaling (Reversat et al., 2015; Yuseff et al., 2011).

The actin protrusions are driven by BLNK-mediated recruitment of the Vav family of guanine nucleotide exchange factors (GEF). Vav2 activates both the Rac and Cdc42 GTPases that act via the WASP and WASP-family verprolin-homologous protein (WAVE) proteins to activate the Arp2/3 complex, leading to rapid outward actin polymerization similar to that seen in lamellipodia formation (Tybulewicz and Henderson, 2009; Kurisu and Takenawa, 2009; Weber et al., 2008). As the B cell membrane begins to contract, dynein motor complexes are recruited to antigen-bound BCR microsignalosomes. They then travel along microtubules towards the MTOC and gathers the signaling clusters into a central supramolecular activation cluster (cSMAC) (Schnyder et al., 2011; Fleire et al., 2006). The cSMAC is surrounded by the actin-rich peripheral SMAC (pSMAC) that is characterized by a high concentration of lymphocyte function-associated antigen 1 (LFA1) and very late antigen 4 (VLA4) integrins that are upregulated in response to BCR signaling. These bind to ICAM-1 and VCAM-1, respectively, on the APC surface to provide stability and support for the synapse (Dustin and Springer, 1988; Carrasco and Batista, 2006). An outermost layer, termed the
distal SMAC, encompasses the cSMAC and pSMAC and contains negative regulators of BCR signaling such as the CD45 tyrosine phosphatases (Abraham et al., 2016). Finally, antigen is extracted from the APC via internalization of antigen-BCR complexes from the cSMAC. These become processed and re-displayed on the B cell to elicit T cell help (Siemasko and Clark, 2001). Therefore, collective use of the actin and microtubule networks enable BCR-induced cell spreading and immune synapse formation which contribute to B cell activation.

### 1.3.4 The Rap GTPases

The Rap GTPases are major regulators of cytoskeleton dynamics (Bos, 2005). In B cells, Rap GTPases have been found to be important for integrin-mediated adhesion, cell spreading, and actin polymerization (McLeod et al., 2004). There are five mammalian Rap proteins: Rap1a, Rap1b, Rap2a, Rap2b, and Rap2c. Both mouse and human Rap1a and Rap1b share 95% sequence similarity while Rap2a, Rap2b, and Rap2c proteins share 90-95% (Paganini et al., 2006). The effector binding regions of both Rap1 and Rap2 families are highly conserved (Heo and Meyer, 2003). BCR binding to antigen can induce cytoskeletal changes through Rap1, which is activated in response to PLCγ2 activation upon BCR signaling (McLeod et al., 1998). PLCγ2 cleaves PIP2 into the second messengers IP3 and DAG. DAG then binds to DAG-regulated GEFs, such as RasGRPs (Stone, 2011) to activate Rap1 (McLeod et al., 1998). Active Rap1 can recruit Vav2 to mediate activation of Rac and Cdc42 (Arthur et al., 2004; Schwamborn and Puschel, 2004), which then activates the Arp2/3 actin-nucleator complex. Similarly, the Cofilin actin-severing protein is transiently de-phosphorylated and becomes active after BCR stimulation. Rap1 has been shown to be important for its activation and interfering with Cofilin dephosphorylation impairs B cell microcluster formation (Freeman et al., 2011). Furthermore, BCR-induced MTOC polarization towards the immune synapse requires Rap1-regulated Cofilin activity (Wang et al., 2017). Depletion of Rap2c, the only Rap2 isoform expressed in the A20 B cell line, also blocks BCR-induced MTOC polarization (Wang et al., 2018), indicating the importance of Rap proteins in the regulation of cytoskeleton-dependent processes in B cells.
1.4 Biomechanical properties of B lymphocytes

All tissues and cells in the body are subject to external physical stimuli. These include gravitational compressive forces that control the deposition of bone (Plotkin et al., 2015), fluid shear stress that arise from the flow of blood (Pruitt et al., 2014), or tensile stresses that regulate vascular permeability (Cines et al., 1998). Cells can sense mechanical forces and they are often integral in the growth, maturation, and function of living tissues (Chen et al., 2004). The ability to sense and convert a mechanical stimuli into an intracellular biochemical signal is termed mechanotransduction (Kaazempur Mofrad et al., 2005). Mechanotransduction in mesenchymal cells such as fibroblasts occur at specialized structures termed focal adhesions that physically link the cellular cytoskeleton to the extracellular matrix (ECM) (Mui et al., 2016) (see more in section 4.2.1). In epithelial cells that are surrounded by neighbouring cells, mechanotransduction also occur at cell-cell junctions termed adherens junctions (Nelson, 2009; Broussard et al., 2017). These junctions are established by the homodimeric interaction of E-cadherin (epithelial cadherin) proteins between adjacent cells. The cytoplasmic domain of cadherins is able to bind to catenin proteins, which interacts with the cortical actin cytoskeleton and stabilizes cadherins at the plasma membrane (Gumbiner, 2005; Takeichi, 2014). Stretching forces directed at the junction causes a tension-dependent conformational change in α-catenin, which recruits vinculin to the cadherin complex (Yonemura et al., 2010; Huveneers et al., 2012; Yao et al., 2014). Vinculin-mediated intracellular signaling then causes increased cell stiffness and alters focal adhesions (Barry et al., 2015). Note that, however, not all forms of mechanotransduction occur at focal adhesions or junctions. Single proteins may act as mechanosensors. For example, stretch-activated ion channels of the Piezo family undergo conformational changes in response to tensile stress (Pathak et al., 2014; Ranade et al., 2014). Immune cells can sense extracellular matrix stiffness by exerting forces onto the substrate (Assoian and Klein, 2008; Judokusumo et al., 2012). This is achieved through myosin II motor-based contractility that is dependent on the actin cytoskeleton (Jannat et al., 2011; Natkanski et al., 2013).
Lymphocytes modulate their contractile forces to exert stronger forces on stiffer substrates and weaker forces on softer ones (Califano and Reinhart-King, 2010; Elosegui-Artola et al., 2016). The B cell immune synapse is also a conduit for intercellular mechanical forces and provides feedback about the strength of the antigen-BCR interaction (Basu and Huse, 2017; Huse, 2017). APCs can differ in their mechanical properties (Bufi et al., 2015). DCs have low cortical membrane tension, and therefore their membranes deform easily (Natkanski et al., 2013). To extract antigen from dendritic cells, B cells tear off pieces of the DC membrane without the need to separate the antigen from the DC membrane. In these cases, the affinity of the BCR less important and B cells will efficiently acquire antigens even with low-affinity BCRs (Spillane and Tolar, 2017). Follicular dendritic cells, in contrast, have high membrane and cytoskeletal tension and thus represent a stiffer substrate (Spillane and Tolar, 2017). Antigens presented on stiff surfaces generate greater tension on the BCR and therefore follicular dendritic cells promote affinity discrimination by the B cell because low-affinity antigens do not maintain strong interactions with the BCR to produce a significant response (Tolar, 2017). This membrane resistance ensures only B cells producing high-affinity BCR-antigen interactions are selected to generate an antibody response (Tolar and Spillane, 2014), and demonstrates one way that the body utilizes biomechanical interactions to aid B cell activation.

1.5 Connexins: Connexin43

1.5.1 Structure, Function, and Assembly

Connexins are highly regulated transmembrane proteins. A total of 21 different connexin genes have been identified in the human genome (Goodenough and Paul, 2009). Each gene encodes proteins that share a common topology consisting of cytoplasmic amino and carboxyl domains and four transmembrane domains (1-4) connected by two extracellular loops (between 1-2, and 3-4) and one cytoplasmic loop (between 2-3) (Figure 1.2A). The carboxyl tail domain (CT) varies in length and is thought to have channel regulatory and protein-interaction functions (Saez et al., 2003; Laird, 2006; Solan and Lampe, 2009). Other families of genes encoding gap junction proteins unrelated to
connexins, called innexins and pannexins, have also been discovered. Innexins encode proteins found in invertebrates including *Drosophila* and *Caenorhabditis* species while pannexins are vertebrate homologs of innexins. Connexins share little sequence similarity with innexins and pannexins but they all have similar structural topology (Phelan and Starich, 2001; Scemes et al., 2009). Connexin43 (Cx43) is the most widely expressed connexin protein *in vivo* and is expressed in most cell lines (Kelly et al., 2015; Leybaert et al., 2017; Laird, 2006). The CT of Cx43 appears to be the primary site of phosphorylation and regulation, but in Cx36 and Cx56 the cytoplasmic loop can also be phosphorylated (Urschel et al., 2006). The Cx43 cytoplasmic loop only contains two threonine residues but no tyrosine or serine residues, and it is unlikely that the amino terminus undergoes phosphorylation given that it does not contain either of these amino acids (Solan and Lampe, 2009), suggesting that the Cx43 CT is the principal domain involved in intracellular processes mediated by phosphorylation.
Figure 1.2. Structure and function of Connexin43.

A: Each Cx43 monomer consists of four transmembrane domains (TM1-4) that are connected by two extracellular loops (EL1 and EL2) and a cytoplasmic loop (CL). The amino terminus (N) and carboxyl tail (CT) and C-terminus (C) extend into the cytoplasm. B: Six Cx43 monomers oligomerize together to form a hexameric complex known as a connexon or hemichannel that is transported to the cell surface, creating a gateway connecting the cytosol to the extracellular milieu. C: Hemichannels may also dock with hemichannels of neighbouring cells to form intercellular communication channels known as gap junctions. These channels aggregate to form gap junction plaques for exchange of ions and second messengers etc.. EC: Extracellular; IC: Intracellular; Ca2+: Calcium ions; IP3: Inositol-1,4,5-trisphosphate. This figure is modified from Ribeiro-Rodrigues et al. *Journal of Cell Science*. 2017 and Neijssen et al. *Progress in Biophysics and Molecular Biology*. 2007.
The association of six identical (homomeric) or different (heteromeric) connexins lead to the formation of a hexameric structure called a hemichannel (also known as a connexon) (Pfenniger et al., 2011) (Figure 1.2B). These hemichannels allow the direct exchange of small molecules between the cytosol and the extracellular environment, thereby playing roles in paracrine communication during disease (Leithe et al., 2018; Begandt et al., 2017; Leybaert et al., 2017). Hemichannels may dock with other connexons of neighbouring cells to form direct intercellular communication channels (Figure 1.2C). Typically, hundreds of thousands of these channels aggregate to form a well-defined gap junction plaque. The plaque typically accumulates through a lateral movement of Cx43 oligomers that have been transported to the plasma membrane (Solan and Lampe, 2014). These gap junctions allow adjacent cells to share ions, small metabolites, second messengers, and other signaling molecules (Goodenough and Paul, 2009; Neijssen et al., 2007) and thereby provides a way for cells to synchronize responses, e.g. heart beats in multicellular organisms (Kanno and Saffitz, 2001). The extracellular loops of connexins contain six cysteine residues that form three disulfide bonds (Rahman et al., 1993). It has been reported that extracellular-Cys-deficient Cx43 expressed in Xenopus laevis oocytes do not form gap junction channels, but are able to form functional hemichannels, suggesting that in oocytes the cysteines are important in gap junction formation (Bao et al., 2004). Moreover, mutations in the amino-terminal domain of Cx43 or in its cytoplasmic loop have been found to render gap junction channels non-functional and contributes to the disease oculodentodigital dysplasia (Kelly et al., 2016; Laird, 2014). Thus, a primary function of Cx43 is in intra- and intercellular communication.

Cx43 assembly begins with co-translational insertion into the endoplasmic reticulum (Ahmad et al., 1999; Laird, 2006) and oligomerization into connexons when it reaches the trans-golgi network (Musil and Goodenough, 1993). Connexons are then packaged into vesicles and trafficked via microtubules to the plasma membrane (Shaw et al., 2007; Laird, 2006). They may be delivered to areas without existing gap junctions in which case the hemichannels will diffuse laterally until they
coalesce with gap junction plaques (Lauf et al., 2002) or they may be delivered directly to existing
gap junctions at cell-cell contact sites (Shaw et al., 2007; Smyth and Shaw, 2012). Gap junctions are
dynamic structures that rapidly disassemble and remodel in response to stress such as wounding
(VanSlyke and Musil, 2005). The Cx43 CT is important in mediating Cx43 endocytosis and
degradation, and this is controlled by various signaling pathways, including phosphorylation by
mitogen-activated protein kinases (Falk et al., 2014b; Leithe, 2016). Internalization of entire gap
junctions can occur whereby one cell takes up the gap junction via formation of a double-membrane
structure known as an annular gap junction or connexosome (Laird, 2006). After internalization, the
connexosome becomes an autophagosome which fuses with lysosomes and is degraded.
Connexosomes may also fuse directly with lysosomes (Leithe et al., 2018). Cx43 has a half-life of 1-3
hours in vivo and in cultured cells and its turnover is considered to be much faster than the average
integral membrane protein (Laird et al., 1991; Lampe, 1994; Beardslee et al., 1998). Modulation of
the connexin turnover rate may be a mechanism by which cells regulate intercellular communication
(Herve et al., 2007). Thus, Cx43 assembly and disassembly is tightly controlled, likely by the CT
domain, to ensure intercellular communication is also tightly regulated.

1.5.2 Channel-independent functions: regulation of cytoskeleton-driven processes

It is now recognized that Cx43, in particular its CT domain, has additional roles such as in
regulation of gene transcription, intracellular signaling, and cytoskeleton-dependent processes, that
are beyond its channel function (Leo-Macias et al., 2016; Esseltine and Laird, 2016; Matsuuchi and
Naus, 2013). Indeed, the Cx43 CT is estimated to be around 150 amino acids long and accounts for
almost 39% of the entire Cx43 protein (Axelsen et al., 2013; Leithe et al., 2018). The Cx43 CT is
important in almost all aspects of the Cx43 life cycle, including trafficking, channel gating, and
localization of gap junction channels (Solan and Lampe, 2009; Solan and Lampe, 2014; Spagnol et
al., 2016b). The structure of the CT is considered to be intrinsically disordered, which encourages
interaction with different molecular partners (Spagnol et al., 2016a). Furthermore, its rich
composition of proline, tyrosine, and serine residues enables the Cx43 CT to be extensively phosphorylated and possess multiple protein interaction motifs such as Src Homology 2 / 3 (SH2 / SH3)-binding domains (Sorgen et al., 2004; Sorgen et al., 2018; Palatinus et al., 2012). Mice lacking Cx43 die at birth due to a cardiac malformation that results in defects to pulmonary gas exchange (Reaume et al., 1995). It was later discovered that this was due to impaired cell migration of cardiac neural crest cells (Huang et al., 1998) that are involved in the normal development of the heart, demonstrating the importance of Cx43 in cytoskeleton-driven cellular processes. Using cardiac neural crest cells obtained from Cx43 knockout mice, Lo and colleagues further found that the motility of these cells was affected by the loss of Cx43 because Cx43-negative cells moved with less directionality and were slower as the concentration of fibronectin increased. In contrast, Cx43+ cells displayed increased directionality and faster locomotion under the same conditions (Xu et al., 2006).

In a separate study involving wound-healing assays with breast epithelial cells, Cx43+ cells traveled uniformly in the same direction towards the wound edge whereas Cx43 knockdown cells moved in sporadic directions, suggesting a significant defect in polarity establishment and directed-migration (Simpson et al., 2008). In particular, the role of the Cx43 CT has been studied extensively in neuronal and glioma cells. These studies established that the Cx43 CT regulates cell motility and directed-migration (Bates et al., 2007; Elias et al., 2007; Cina et al., 2009), with the CT alone being sufficient to promote glioma cell motility (Crespin et al., 2010) independently of its channel function (Sin et al., 2016). Cell motility and migration is critically dependent on the ability to reorganize the cellular cytoskeleton (Blanchoin et al., 2014). Therefore, Cx43’s importance extends beyond channel communication and suggests its CT could be a major regulator of cellular processes involving cytoskeleton dynamics.

1.5.3 Connexin43 in the immune system

Connexin43 is the predominant connexin expressed in immune cell types (Neijssen et al., 2007). Gap junction formation between monocytes and macrophages is upregulated upon exposure to
inflammatory factors (Eugenin et al., 2003), and Cx43 and Cx37 expressed on neutrophils and monocytes may form hemichannels to mediate ATP release (Eltzschig et al., 2006; Evans et al., 2006; Wong et al., 2006). Activated lymphocytes have also been shown to form gap junctions with endothelial cells during extravasation (Bopp et al., 2007; Fonseca et al., 2004). Cx43 and Cx40 appear to be the only connexin proteins expressed in T and B lymphocytes (Oviedo-Orta et al., 2000). Reverse transcription polymerase chain reactions and western blotting detected Cx43 mRNA and protein, respectively, in circulating peripheral blood and tonsil-derived lymphocytes from patients with recurrent tonsillitis. Cx40 was only found in tonsil-derived samples (Oviedo-Orta et al., 2000). Cx43 may allow B cells to interact via gap junction channels with follicular dendritic cells encountered in secondary lymphoid organs, which could help facilitate transfer of pro-survival signals for the B cell (Krenacs et al., 1997). However, less is known about non-channel functions of Cx43 in B cells.

1.6 Rationale: the role of Cx43 in B cell cytoskeleton-dependent processes

The Matsuuchi lab had previously found that Cx43 expression in Cx43-negative J558µm3 plasmacytoma B cells was sufficient to restore/enhance BCR-mediated cell spreading on glass coverslips with immobilized anti-BCR antibodies. Cx43-negative J558µm3 cells do not normally sustain a BCR-mediated spreading response (Machtaler et al., 2011). Because cell spreading relies on actin cytoskeleton remodeling (Fleire et al., 2006), this finding prompted the realization that Cx43 may be a critical piece in the pathway enabling cytoskeleton-driven morphological alterations in B cells. This promotion was dependent on Rap1 activation (Falk et al., 2014a), suggesting that Cx43 influenced intracellular signaling. Further studies showed that the knockdown of Cx43 impaired cell motility and directed-migration towards a chemokine gradient in WEHI231 B cells (Machtaler et al., 2014). Meanwhile, Cx43 knockdown in WEHI231 cells also disrupted the activation of various BCR signaling proteins (Choi, 2012). Loss of the Cx43 CT domain resulted in overall decreased B cell adhesion to an endothelial cell monolayer, an inability to maintain a BCR-mediated cell spreading
response on immobilized anti-BCR antibodies, and an attenuated BCR-induced Rap1 GTPase activation (Machtaler et al., 2011). These findings strongly indicated that the Cx43 CT domain could be a key regulator of B cell cytoskeleton-dependent processes.

1.7 Objectives

The objective of this thesis was to investigate the role of Cx43 and its CT in B cell cytoskeleton dynamics. We hypothesized that Cx43’s effect on cytoskeletal changes is through influencing receptor signaling-induced cytoskeleton remodeling, such as by affecting the activity of BCR pathway-associated proteins that modify the cytoskeleton. As BCR signaling induces cytoskeletal changes, we further hypothesized that the Cx43 CT influences overall BCR signaling responses. Various Cx43 CT mutant constructs had previously been transfected into BCR-positive, Cx43-negative J558μm3 cells by the Matsuuchi lab. The aim was to use these mutants to gain insight into the importance of the Cx43 CT in BCR signaling.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Antibodies

2.1.1.1 Antibodies for cell stimulation or cell attachment

Polyclonal goat anti-mouse IgM (\(\mu\) chain specific, #115-005-020) and goat anti-mouse IgG (Fc\(\gamma\) fragment specific, #115-005-008) used for cell stimulation (section 2.2.2) and cell spreading (section 2.2.6) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Monoclonal mouse anti-mouse MHC class I (clone 34-1-2S, #14-5998-81) antibodies used in cell stimulation (section 2.2.2), flow cytometry (section 2.2.4), and membrane stretching experiments (section 2.2.5.2) were from ThermoFisher Scientific eBioscience (Waltham, MA, USA). Monoclonal mouse anti-DNP (Dinitrophenyl) (IgE isotype, clone SPE-7, #D8406) antibodies used to coat mast cells prior to cell stimulation (section 2.2.5.1) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.1.2 Antibodies for Western blotting

Primary antibodies used in western blotting are summarized in Table 2-1. The pan-species anti-phosphotyrosine monoclonal antibody (clone 4G10, Gold et al., 1990) was prepared by May Dang-Lawson in the Matsuuchi lab. Briefly, 4G10 hybridomas were grown in cell culture media containing 1% glucose, 25 mM HEPES, 5 mM NaOH. Confluent cultures were left until cells died and culture media containing antibody was collected and filter sterilized.

<table>
<thead>
<tr>
<th>Antibody recognizes</th>
<th>Dilution used</th>
<th>Host (formulation)</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
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<td>(\beta)-actin</td>
<td>1:1500</td>
<td>Polyclonal rabbit</td>
<td>Invitrogen</td>
<td>#PA5-16914</td>
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<tr>
<td>Phospho-Akt (Ser473)</td>
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<td>Polyclonal rabbit</td>
<td>CST</td>
<td>#9271</td>
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<tr>
<td>Akt</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST</td>
<td>#9272</td>
</tr>
<tr>
<td>Phospho-BLNK (Tyr96)</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST</td>
<td>#3601</td>
</tr>
<tr>
<td>BLNK</td>
<td>1:1000</td>
<td>Monoclonal rabbit</td>
<td>CST</td>
<td>#12168</td>
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<td>Phospho-Btk (Tyr223)</td>
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<td>Polyclonal rabbit</td>
<td>CST</td>
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<tr>
<td>Btk</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST</td>
<td>#3532</td>
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<td>Species</td>
<td>Catalog Number</td>
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<td>----------</td>
<td>------------------</td>
<td>----------------</td>
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</tr>
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<tr>
<td>CD79α</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST #3351</td>
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<tr>
<td>Phospho-Cofilin (Ser3)</td>
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<td>Polyclonal rabbit</td>
<td>CST #3313</td>
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<tr>
<td>Cofilin</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST #3318</td>
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<td>Polyclonal rabbit</td>
<td>Sigma-Aldrich</td>
<td>#C6219</td>
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<td>Monoclonal rabbit</td>
<td>CST #4377</td>
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<tr>
<td>p44/42 MAPK (ERK1/2)</td>
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<td>Polyclonal rabbit</td>
<td>CST #9102</td>
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<td>CST #3141</td>
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<td>Phospho-FER (Tyr402)</td>
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<td>Polyclonal rabbit</td>
<td>Abbexa #ax012599</td>
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<td>GFP</td>
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<td>Polyclonal rabbit</td>
<td>Synaptic Systems</td>
<td>#132002</td>
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<td>HS1</td>
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<td>Polyclonal rabbit</td>
<td>CST #4557</td>
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<tr>
<td>Lyn</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST #2732</td>
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<td>Phospho-PLCy2 (Tyr1217)</td>
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<td>PLCy2</td>
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<td>Polyclonal rabbit</td>
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<td>Phospho-Pyk2 (Tyr402)</td>
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<td>Pyk2</td>
<td>1:1000</td>
<td>Polyclonal rabbit</td>
<td>CST #3292</td>
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<td>Polyclonal rabbit</td>
<td>CST #2701</td>
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<td>Syk</td>
<td>1:1000</td>
<td>Monoclonal rabbit</td>
<td>CST #13198</td>
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<tr>
<td>Phospho-tyrosine (4G10)</td>
<td>1:2000</td>
<td>Monoclonal mouse</td>
<td>Matsuuchi lab</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Polyclonal goat anti-rabbit IgG (H + L) (#170-6515) and goat anti-Mouse IgG (H + L) (#170-6516) conjugated to horseradish peroxidase (HRP) secondary antibodies were purchased from Bio-Rad (Mississauga, ON, Canada). Purified mouse IgG-kappa-binding protein conjugated to HRP (Santa Cruz Biotechnology) was also used to detect primary antibodies. These proteins are listed in table 2-2.

**Table 2-2. Secondary antibodies used for Western blotting**

<table>
<thead>
<tr>
<th>Antibody recognizes</th>
<th>Conjugation</th>
<th>Dilution used</th>
<th>Host (formulation)</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>HRP</td>
<td>1:3000</td>
<td>Goat</td>
<td>Bio-Rad</td>
<td>#170-6515</td>
</tr>
<tr>
<td>Mouse IgG (H+L)</td>
<td>HRP</td>
<td>1:3000</td>
<td>Goat</td>
<td>Bio-Rad</td>
<td>#170-6516</td>
</tr>
<tr>
<td>Mouse IgG kappa light chain</td>
<td>HRP</td>
<td>1:1000</td>
<td>N/A</td>
<td>SCBT</td>
<td>#sc-516102</td>
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</tbody>
</table>

**Bio-Rad:** Bio-Rad (Mississauga, ON, Canada; [http://www.bio-rad.com/](http://www.bio-rad.com/)); **SCBT:** Santa Cruz Biotechnology (Dallas, TX, USA; [https://www.scbt.com/scbt](https://www.scbt.com/scbt))

### 2.1.1.3 Antibodies for flow cytometry analysis

Monoclonal rat anti-mouse IgM conjugated to Phycoerythrin (PE) (clone II/41, #12-5790-81) was purchased from ThermoFisher Scientific eBioscience. Monoclonal rat anti-mouse MHC Class II FITC-conjugated antibody (clone M5/114.15.2, #11-5321-81) was from ThermoFisher Scientific eBioscience. Alexa Fluor® 488-conjugated polyclonal goat anti-mouse IgG (H+L) (#A-11029) was from ThermoFisher Scientific Invitrogen (Waltham, MA, USA). Rat anti-mouse CD16/CD32 (clone 2.4G2) was prepared by May Dang-Lawson in the Matsuuchi lab.

### 2.1.2 Plasmids

The base AP2 expression vector and NAP2 expression vectors encoding cDNA for rat wild-type (WT) Cx43 with enhanced green fluorescent protein (EGFP) fused in-frame to the end of the Cx43 carboxyl tail (CT) (Cx43-EGFP) or cDNA for Cx43 with its CT truncated at aa246 and fused to EGFP (ΔCT246 Cx43) were obtained from Dr. Christian Naus (Dept. Cellular and Physiological Sciences, Life Sciences Institute, UBC, Vancouver, Canada). AP2 is a murine retroviral vector.
originally designed by the Nalbantoglu Lab (McGill University, Montreal, Canada) (Galipeau et al., 1999). It contains a multiple cloning site (MCS) under Cytomegalovirus (CMV) promoter control and an enhanced green fluorescence reporter gene under internal ribosome entry site (IRES) control (Galipeau et al., 1999). NAP2 is derived from the AP2 vector and was created in the Naus lab by using endonuclease digestion to remove cDNA fragments coding for IRES and EGFP followed by insertion of Cx43-EGFP or ΔCT246 Cx43 and has been described previously in Mao et al., 2000 and Fu et al., 2004, respectively.

2.1.3 Cell lines

J558μm3 is a terminally-differentiated myeloma (i.e. plasmacytoma) murine B cell line in which the BCR has been reconstituted by transfection of 3 of the receptor chains (μ heavy chain, Ig-α, and Ig-β - λ light chain is endogenously expressed) (Reth et al., 1987; Hombach et al., 1988). These cells were a gift from Dr. Louis Justement (University of Alabama, Birmingham, AL, USA) (Justement et al., 1990) and have been used previously in the literature as a model to study the BCR signaling pathway (Hombach et al., 1990; Justement et al., 1991; Adachi et al., 2001). WEHI231 is an immature murine IgM+ B lymphoma cell line (Jakway et al., 1986; Gold et al., 1990) and A20 cells are murine IgG+ mature B lymphoma cells (Kim et al., 1979; Law et al., 1992). Both of these lines were obtained from the American Type Culture Collection (ATCC, #CRL-1702 and #TIB-208, respectively) and are commonly used in the Gold and Matsuuchi labs (Machtaler et al., 2011; McLeod et al., 1998; Wang et al., 2017).

2.1.4 J558μm3 Cx43 mutants used in this study

2.1.4.1 DNA preparation

NAP2 vectors containing Cx43-EGFP, ΔCT246 Cx43 were described in a previous section (section 2.1.2). Creation of ΔCT258 Cx43 and ΔCT307 Cx43 mutant DNA in which the CT of Cx43 is truncated at aa258 and aa307, respectively was done by Farnaz Pournia using the NAP2 vector containing Cx43-EGFP as template and is described in detail in her M.Sc. thesis (Pournia, 2015).
Additional Cx43 CT mutants with individual serine-to-alanine point mutations (S255A, S262A, S279A, S282A Cx43) as well as a mutant with all four serines mutated to alanine simultaneously (4S Cx43) were made by May Dang-Lawson and Farnaz Pournia, respectively, using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON, Canada, #210518) on a Cx43-EGFP backbone.

2.1.4.2 DNA transfection

J558µm3 were transduced with Cx43 plasmid DNA using a retroviral transfection protocol based on the BOSC23 retroviral packing cell line (Pear et al., 1993). Briefly, plasmid DNA was introduced into BOSC23 by calcium phosphate precipitation transfection (Krebs et al., 1999) and culture media containing virus was collected after 24, 48 and 72 h of initial transfection. This was passed through 0.45 μm filters (VWR, Mississauga, ON, Canada) and used to suspend 0.5 x 10⁶ J558µm3 cells for infection. After 2-3 days, J558µm3 cells were sorted by fluorescence-activated cell sorting (FACS) (section 2.2.4) for high expression of EGFP and IgM. A summary of the J558µm3 Cx43 mutants used in this study is shown in Table 2-3. Correct expression and membrane localization of the Cx43 proteins in these mutants have been documented (Pournia, 2015).

Table 2-3. Cx43 mutants used in this study

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Description</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP2</td>
<td>Expression vector encoding for EGFP</td>
<td>Dr. Christian Naus (UBC, Vancouver, Canada)</td>
<td>Galipeau et al., 1999</td>
</tr>
<tr>
<td>Cx43-EGFP</td>
<td>Cx43 with EGFP fused in-frame to CT in NAP2 vector</td>
<td>Dr. Christian Naus (UBC, Vancouver, Canada)</td>
<td>Mao et al., 2000</td>
</tr>
<tr>
<td>ACT246 Cx43</td>
<td>Cx43 with its CT truncated at aa246 and fused to EGFP in NAP2 vector</td>
<td>Dr. Christian Naus (UBC, Vancouver, Canada)</td>
<td>Fu et al., 2004</td>
</tr>
<tr>
<td>ACT258 Cx43</td>
<td>Cx43 with its CT truncated at aa258 and fused to EGFP in NAP2 vector</td>
<td>Made by Farnaz Pournia (Matsuuchi lab)</td>
<td>Pournia, 2015</td>
</tr>
</tbody>
</table>
Table 2-3. Cx43 mutants used in this study (cont.)

<table>
<thead>
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<th>Cx43 Mutant</th>
<th>Description of Mutant</th>
<th>Made by</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT307 Cx43</td>
<td>Cx43 with its CT truncated at aa307 and fused to EGFP in NAP2 vector</td>
<td>Farnaz Pournia (Matsuuchi lab)</td>
<td>Pournia, 2015</td>
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<td>S255A Cx43</td>
<td>Cx43 with a serine-to-alanine point mutation at aa S255 of its CT. Fused to EGFP and in NAP2 vector</td>
<td>May Dang-Lawson (Matsuuchi lab)</td>
<td>Unpublished²</td>
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<tr>
<td>S262A Cx43</td>
<td>Cx43 with a serine-to-alanine point mutation at aa S262 of its CT. Fused to EGFP and in NAP2 vector</td>
<td>May Dang-Lawson (Matsuuchi lab)</td>
<td>Unpublished²</td>
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<tr>
<td>S279A Cx43</td>
<td>Cx43 with a serine-to-alanine point mutation at aa S279 of its CT. Fused to EGFP and in NAP2 vector</td>
<td>May Dang-Lawson (Matsuuchi lab)</td>
<td>Unpublished²</td>
</tr>
<tr>
<td>S282A Cx43</td>
<td>Cx43 with a serine-to-alanine point mutation at aa S282 of its CT. Fused to EGFP and in NAP2 vector</td>
<td>May Dang-Lawson (Matsuuchi lab)</td>
<td>Unpublished²</td>
</tr>
<tr>
<td>4S Cx43</td>
<td>Cx43 with all four S255A, S262A, S279A, S282A serine-to-alanine point mutations in its CT. Fused to EGFP and in NAP2 vector</td>
<td>Farnaz Pournia (Matsuuchi lab)</td>
<td>Unpublished²</td>
</tr>
</tbody>
</table>

1—All Cx43 mutants were transfected and expressed in J558um3 plasmacytoma cell lines that are endogenously Cx43-negative.
2—Please note that a full description of these mutants is in preparation in Pournia et al., 2018 (Pournia, F., Dang-Lawson, M., Choi, K., Mo, V., Lampe, P.D., and Matsuuchi, L. (2018). Single, double, and multiple serine mutations within a key region of Cx43 CT have distinct effects on BCR-mediated cell spreading of B-lymphocytes.)

2.2 Methods

2.2.1 Cell culture

Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, #R0883) supplemented with 5% heat inactivated fetal bovine serum (HI-FBS) (ThermoFisher Scientific Invitrogen, #12483-020), 2 mM L-glutamine (Sigma-Aldrich, #G-8540), 1 mM sodium pyruvate (Sigma-Aldrich, #P5280), 50 units/ml penicillin (ThermoFisher Scientific...
Invitrogen, #15140122), 50 μg/ml streptomycin (ThermoFisher Scientific Invitrogen, #15140122), and 50 μM β-mercaptoethanol (2-ME) (Sigma-Aldrich, #M7154). Cells were maintained in a direct heat incubator (ThermoForma, Waltham, MA, USA) at constant 37°C and 5% CO₂ and passaged as needed to be at approximately 5 – 7 x 10⁵ cells/ml. Frozen stocks for long-term storage were made by re-suspending approximately 1.5 x 10⁶ cells in 500 µl FBS plus 10% dimethyl sulfoxide (DMSO) (MP Biomedical, Santa Ana, CA, USA, #191418) and storing at -80°C for a three days before putting in liquid nitrogen storage.

2.2.2 Cell stimulation and preparation of lysates

Cells were spun down at 1500 RPM for 5 min in an Allegra X-14R Centrifuge (Beckman Coulter Inc., Brea, CA, USA) to be washed once with and re-suspended at approximately 8 - 9 x 10⁶ cells/ml in 400 µl modified HEPES buffered saline (25 mM sodium HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 0.5 mM MgSO₄, 1 g/l glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, adjusted to pH 7.2) followed by incubation for 10 min on ice and 20 min in a 37°C water bath. Cells were then stimulated with either 20 μg/ml goat anti-mouse IgM (Jackson ImmunoResearch Labs, #115-005-020), 20 μg/ml goat anti-mouse IgG (Jackson ImmunoResearch Labs, #115-005-008), or 3 μg/ml mouse anti-mouse MHC class I (ThermoFisher, #14-5998-81) antibodies in solution for the indicated times and stopped with equal volumes cold phosphate-buffered saline (PBS) (ThermoFisher Scientific Gibco, #10010-023) containing 1 mM Na₃VO₄. Cells were then spun at 2800 RPM for 4 min and the pellet lysed with cold radioimmunoprecipitation assay (RIPA) buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL (Sigma-Aldrich, #CA-630), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM Ethylenediaminetetraacetic (EDTA), and protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml β-Glycerophosphate, 10 μg/ml sodium molybdate, 1 μg/ml aprotinin). Samples were vortexed for 10 seconds, left on ice for 15 min, and centrifuged at 14,000 RPM at 4°C for 15 min to pellet
cellular debris. Protein concentration was determined using a bicinchoninic acid (BCA) assay (ThermoFisher Scientific Pierce, Rockford, IL, USA #23225) with an Ultrospec 2011 pro spectrophotometer (Sigma-Aldrich GE Healthcare, Chicago, IL, USA, #80-2112-21) and lysates were subsequently diluted 1:5 with 5x reducing sample buffer (62.5 mM Tris, 2% glycerol, 2% SDS, 100 mM DTT pH 6.8, 0.02% bromophenol blue) and boiled for 5 minutes for gel loading immediately or storage at -80°C.

Mild lysis conditions and sonication were instead employed (Machtaler et al., 2011) if the intention was to probe for Cx43 in western blot. Cells were similarly washed and suspended in HEPES buffered saline and incubated on ice for 10 min and in a 37°C water bath for 20 min before lysis in cold modified K buffer (50 mM CaCl₂, 1% Triton-X 100, 1% IGEPAL in PBS) (Troxell et al., 1999) containing protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml β-Glycerophosphate, 10 µg/ml sodium molybdate, 1 µg/ml aprotinin. Samples were then sonicated for 10 seconds at level ‘3.5’ using the Misonix XL Sonicator Ultrasonic Cell Processor (Misonix Incorporated, Farmingdale, NY, USA) and immediately put on ice for 15 min. Afterwards, samples were centrifuged at 14,000 RPM at 4°C for 15 min to pellet cellular debris and protein concentration was determined using the BCA assay. Finally, lysates were diluted 1:5 with 5x reducing sample buffer (62.5 mM Tris, 2% glycerol, 2% SDS, 100 mM DTT pH 6.8, 0.02% bromophenol blue) and incubated in a 37°C water bath for 1 h before proceeding directly to western blot analysis (section 2.2.3).

2.2.2.1 Rap1 Activation Assays

Rap1 activation assays have been described in detail by McLeod et al., 1998. In brief, cells were stimulated and lysates prepared in a similarly manner as outlined above, except that cells were directly lysed in solution by adding equal volumes of 2x Rap lysis buffer (10% glycerol, 1% IGEPAL, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2 mM MgCl₂ supplemented with protease
inhibitors: 1 mM PMSF, 1 mM Na$_3$VO$_4$, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin) without centrifugation before lysis. Ral Guanine Nucleotide Dissociation Stimulator (RalGDS) proteins conjugated to Glutathione S-Transferase (GST) were mixed with Glutathione-Sepharose 4B beads (Sigma-Aldrich GE Healthcare, #17-0756-01) and the prepared lysates were later added to this mixture to selectively pull down GTP-bound Rap1. Beads bound to active Rap1 were then eluted with 1x Rap lysis buffer (1:2 dilution of 2x Rap1 lysis buffer in water) and re-suspended in 30 µl 1x reducing sample buffer and proceeded directly to western blot analysis (section 2.2.3) using anti-Rap1 antibodies (Table 2-1).

2.2.3 Immunoblotting and analysis

2.2.3.1 Western blotting

Sample preparation: lysates were re-boiled for 5 min if they had been stored at -80°C prior (section 2.2.2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as follows. 12% SDS polyacrylamide (Bio-Rad #161-0144) gels were prepared according to standard lab procedures and 25 µg protein/lane were loaded alongside BLUelf prestained protein ladder (FroggaBio, Toronto, ON, Canada, # PM008-0500) to estimate molecular weight. Gels were run in a dual vertical mini-gel apparatus with water-cooling system (Fisher CBS Scientific, Del Mar, CA, USA) in running buffer (50 mM Tris, 0.4 M glycine, 0.1% SDS) at a constant 130 V for 1.5-2 hr.

Separated proteins in the gel were transferred onto nitrocellulose membranes (Bio-Rad, #162-0115) using a Mini Trans-Blot® transfer apparatus containing transfer buffer (20 mM Tris-HCl, 150 mM glycine, 20% methanol) at a constant voltage of 100 V for 1-2 h or 25 V overnight (if probing with anti-phosphotyrosine antibody (4G10) afterwards). The presence of transferred proteins on the membrane was confirmed by soaking the membranes in 0.1 % Ponceau S (Sigma-Aldrich, # P7170) for 30 seconds before rinsing with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 8, 150 mM NaCl) for 5 min or until Ponceau has been washed off. Membranes were then blocked in TBS.
containing 5% (w/v) bovine serum albumin (BSA) or 5% (w/v) skim milk powder (only in the case of probing for Rap1) for 30 min with shaking at room temperature. Milk was not used to block membranes intended for probing with phospho-specific antibodies because milk contains the phosphoprotein casein, which anti-phospho antibodies could bind to non-specifically and cause background bands. Afterwards, primary antibody suspended in TBS containing 5% (w/v) BSA were added for incubation overnight at 4°C or 1 h at room temperature (in the case of probing with anti-phosphotyrosine). Membranes were then washed three times for 10 min each with TBST (TBS plus 0.1% Tween 20) before incubation with HRP-conjugated secondary antibodies (Table 2-1) suspended in TBS plus 5% (w/v) skim milk powder for 1 h at room temperature with shaking. Unbound antibodies were removed with three successive 10 min TBST washes and membranes were kept in TBS until development.

Western blots were developed by applying either enhanced chemiluminescence (ECL) reagent from Millipore-Sigma (Etobicoke, Ontario, Canada # WBKLS0500), or Azure Biosystems (Dublin, CA, USA #AC2101) to the nitrocellulose membrane, and incubating for 2 min before scanning on a C-digit blot scanner (Li-cor, Lincoln, NE, USA). In cases where re-probing was needed, membranes were incubated with antibody stripping buffer (25 mM HCl in TBST) for 45 min at room temperature with shaking and washed with TBS for 20 min before incubating with another primary antibody (Table 2-1).

2.2.3.2 Analysis of Western blots and quantification

Protein bands were assessed and quantified using Image Studio Lite (Li-cor). To obtain a trend of total band intensities over time (e.g. in Figure 3.9e), band intensities of each lane were measured and normalized to their own respective loading control densities. Afterwards, the unstimulated J558µm3 WT Cx43 sample was chosen as a reference and all normalized intensities of Cx43-negative or mutant Cx43 samples were further normalized to this reference so that all bands
became comparable to this control, which now has a baseline value of 1. The adjusted relative
densities from three experiments were averaged and plotted with GraphPad Prism 7 software
(GraphPad Software, La Jolla, CA, USA). Band densities were in the linear range for quantification
and not at density saturation. Density tracings of representative blots plotted against molecular
weights (e.g. in Figure 3.9f) were done by obtaining a density profile tracing of selected bands of the
15-minute time point in Image Studio Lite (Li-cor). Tracings for each cell type were individually re-
traced, coloured, and overlaid with Adobe Illustrator CS6 (Adobe Systems Incorporated, San Jose,
CA, USA).

2.2.4 Flow cytometry

2.2.4.1 Sample preparation

The expression levels of surface proteins were measured by cell surface staining followed by
flow cytometry. Approximately 1 x 10⁶ cells were spun at 1500 RPM for 5 min, washed once with
PBS, and re-suspended in 50 µl of 2% FACS buffer (PBS containing 2% HI-FBS) in 96-well round-
bottom plates (BD Biosciences, Mississauga, ON, Canada, #351172). The Fc receptor blocking
reagent, anti-mouse CD16/CD32 (clone 2.4G2), was then added at 25 µg/ml for 10 min on ice. Cells
were then incubated with 2 µg/ml of the appropriate antibodies (section 2.1.1) for 30 min on ice in
the dark. Afterwards, an additional 100 µl of FACS buffer was added and cells undergo three
successive washes in 150 µl cold FACS buffer. In the case of using anti-mouse IgM-PE
(ThermoFisher, #12-5790-81) or anti-mouse MHC Class II-FITC (ThermoFisher, #11-5321-81), cells
were immediately re-suspended in 300 µl of cold FACS buffer in polystyrene tubes (BD Biosciences,
#352054) on ice shielded from light and brought to the UBC Flow Cytometry Facility (Life Sciences
Institute, UBC, Vancouver, Canada, www.ubcflow.ca). If anti-mouse MHC class I (ThermoFisher,
#14-5998-81) antibodies were used, cells were instead re-suspended in 50 µl of cold FACS buffer and
further incubated with 10 µg/ml Alexa Fluor® 488-conjugated anti-mouse IgG (H+L) (ThermoFisher,
#A-11029) secondary antibodies for 30 min on ice in the dark. Similar washes three times with 150 µl
cold FACS buffer were done before re-suspending in 300 µl cold FACS buffer in polystyrene tubes on ice and brought to the UBC Flow Cytometry Facility.

2.2.4.2 Sample acquisition and analysis

Samples were examined on a BD LSRII Flow Cytometer (BD Biosciences) using the BD FACS Diva software (BD Biosciences) or sorted by the UBC Flow Cytometry Facility on a BD FACS Aria IIu and BD Influx instruments (BD Biosciences). The live cell population was gated and fluorescent intensities corresponding to the expression levels of surface proteins on these cells were plotted using the FlowJo® flow cytometry analysis software (Tree Star Inc., Ashland, OR, USA).

2.2.5 Application of mechanical force to cells

2.2.5.1 Turbulence and shear-induced cell stimulation

Cells were spun down at 1500 RPM for 5 min to be washed once with and re-suspended at approximately 8 - 9 x 10⁶ cells/ml in 400 µl modified HEPES buffered saline (25 mM sodium HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 0.5 mM MgSO₄, 1 g/l glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 µM 2-ME, adjusted to pH 7.2) followed by incubation for 10 min on ice and 20 min in a 37°C water bath. Samples were then inverted up and down (“turbulence”) four times to simulate mechanical disturbances or passaged through a 26G syringe needle (BD Biosciences, #305115) four times to mimic the experience of shear forces and put back in 37°C for 5 min. Mast cells coated with 1 µg/ml anti-DNP IgE (Sigma-Aldrich, #D8406) overnight prior to experiments were also stimulated with 100 ng/ml DNP-BSA (BSA-2,4-Dinitrophenylated) (ThermoFisher Scientific Invitrogen, #A23018) and put back in 37°C for the indicated times. Since the intention was to assess Rap1 activation, cells were then lysed in solution by gentle addition of equal volumes of 2x Rap lysis buffer (10% glycerol, 1% IGEPAL, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2mM MgCl₂ supplemented with protease inhibitors: 1 mM PMSF, 1 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin) and proceeded to further Rap pull-down and western blotting procedures as outlined in sections 2.2.2.1 and 2.2.3.
2.2.5.2 Membrane stretching

Membrane stretching has been performed previously in the Roskelley Lab (Life Sciences Institute, UBC, Vancouver, Canada) (Poon, 2015; Freeman et al., 2016) as well as the Gold lab (Christian, 2017; Wang et al., 2017) and the following protocol was derived from these resources with modifications: untreated BioFlex® six-well culture plates with flexible silicone elastomer bottoms (Flexcell® International, Burlington, NC, USA, #BF-3001U) were washed twice with PBS and incubated with 2.5 ml of PBS containing anti-mouse MHC class I (ThermoFisher, #14-5998-81) antibodies at a final concentration of 1.3 µg/ml (i.e. 0.33 µg/cm² given that each well is 9.62 cm²) with shaking at 4°C overnight. On the next day, cells were first spun down at 1500 RPM for 5 min and washed with PBS once before being suspended in serum-free RPMI-1640 medium (section 2.2.1). Meanwhile, BioFlex® culture plates were washed three times with PBS to remove unbound anti-MHC I antibodies and 2 ml of PBS was added back. Both cells and plates were then incubated at 37°C for 20 min. Afterwards, 2.5 ml of the serum-free RPMI-1640 medium containing approximately 2 x 10⁶ cells was added to the wells of the BioFlex® culture plates and incubated for 15 min at 37°C to let cells settle.

BioFlex® culture plates containing cells were then brought to a BioFlex® Baseplate Kit (Flexcell® International, #BFBK-4000) and fitted into its designated 25 mm cylindrical Arctangle® Loading Posts to be fastened in place with BioFlex® gaskets (Flexcell® International, #G-3000T). The Flexcell® FX-5000™ Tension System (Flexcell® International, #FX-5000T) was then used to generate vacuum pressure which drags the flexible silicone wells downward and expanding it against the cylindrical posts, thereby applying equibiaxial stretch at a chosen magnitude and duration at 37°C on the portion of silicone above the posts.

After the allotted duration of stretching, cells were fixed directly in its well by adding 16% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, USA, #15710) in a 1:4
dilution to a final concentration of 4% for 15 min while cells were held stretched at 37°C. If 7-Aminoactinomycin D (7-AAD) (Millipore-Sigma, #129935) was used to determine live / dead cell states, plates were first taken out of the BioFlex® Baseplate Kit after stretching, media removed gently, and 2 ml of 7-AAD that is diluted 1:1000 in PBS was added to stain for 10 min in the dark at room temperature. Cells were then washed gently with PBS twice and fixed in 2 ml of 4% PFA for 15 min at 37°C. After fixing, cells were again washed gently three times with PBS and a piece of the silicone well (approximately ¼ of the well) was cut out to undergo staining and immunofluorescence imaging procedures outlined in section 2.2.7.

2.2.6 B cell receptor-mediated cell spreading

B cell receptor-mediated cell spreading assays have been described in detail previously (Lin et al., 2008; Machtaler et al., 2011; Pouria, 2015). Briefly, 12 mm glass coverslips or silicone pieces (of approximately the same size) cut from BioFlex® culture plates (Flexcell® International #BF-3001U) were washed twice with PBS and incubated with 200 µl of PBS containing 15 µg/ml (2.5 µg/cm²) anti-mouse IgM (Jackson ImmunoResearch Laboratories, #115-005-020) antibodies with shaking at 4°C overnight to immobilize antibodies onto the surface. The next day, cells were washed once with PBS and re-suspended in serum-free RPMI-1640 medium while substrates (glass or silicone) were washed three times with PBS to remove unbound antibodies. Both cells and substrates were then put in 37°C for 20 min before 100 µl of approximately 2.5 – 5 x 10⁵ cells were added onto the substrates in 37°C according to a time course. On reaching the last time point, cells were fixed directly with 100 µl 8% PFA (to reach a final concentration of 4%) for 15 min at room temperature. After fixing, cells were washed gently with PBS three times and underwent staining and immunofluorescence imaging procedures outlined in section 2.2.7.
2.2.7 Immunofluorescence microscopy

2.2.7.1 Sample preparation and staining

Sample preparation prior to cell staining is outlined in sections 2.2.5.2 and 2.2.6. After cells were fixed, residual PBS was removed by gentle pipette suction, and cells were permeabilized with 200 µl of 0.25% Triton X-100 (Fisher Scientific, #BP15) for 3 min at room temperature. Samples were then gently washed three times with PBS before incubating with Rhodamine Phalloidin (ThermoFisher Scientific Invitrogen, #R415) or Alexa Fluor™ 488-conjugated Phalloidin (ThermoFisher Scientific Invitrogen, #A12379) at a 1:200 dilution in PBS for 30 min at room temperature in the dark to stain for F-actin. Afterwards, cells were washed three times gently with PBS to remove unbound stains and mounted onto 3 x 1" glass microscope slides using ProLong Gold anti-fade reagent supplemented with DAPI (ThermoFisher Scientific Invitrogen, #P36935). Samples were sealed with nail polish and stored at -20°C (coverslips) or 4°C (silicone) prior to imaging.

In experiments that aimed to detect bound anti-mouse MHC class I (ThermoFisher, #14-5998-81) antibodies on silicone, samples were prepared similarly as in section 2.2.5.2 except only 2 x 10⁴ – 5 x 10⁵ cells were incubated onto silicone and all samples were blocked with 200 µl of PBS plus 2% BSA for 30 min at room temperature prior to staining with 200 µl of PBS containing a mixture of 1:200 Rhodamine Phalloidin (ThermoFisher Scientific Invitrogen, #R415) and 1:300 Alexa Fluor® 488-conjugated anti-mouse IgG (H+L) (ThermoFisher, #A-11029) secondary antibody for 30 min at room temperature. Samples were then washed and mounted onto glass microscope slides exactly as outlined above.

2.2.7.2 Image acquisition and quantification

Substrate-cell contact site images were acquired using the 100x oil objective lens on the III-Zeiss spinning disk confocal microscope (LSI imaging facility, UBC, Vancouver, Canada) with SlideBook 6 digital microscopy software (Intelligent Imaging Innovations, Denver, CO, USA) to
focus on the morphology and actin structures of individual cells at high magnification. Image pseudocolouring and addition of scale bars were done with ImageJ image processing software (National Institutes of Health, Bethesda, MD, USA). 3D reconstructions were prepared by stacking together 15 - 20 image slices taken at 0.2 \( \mu \text{m} \) intervals, using SlideBook 6 (Intelligent Imaging Innovations). Cell areas (\( \mu \text{m} \)) of approximately 30-100 cells, from three independent experiments, were acquired by highlighting cells in an image and using the “measure” function in ImageJ (National Institutes of Health). These values were plotted using GraphPad Prism 7 (GraphPad Software).

Detection of bound anti-mouse MHC class I was done using the 20x objective lens on a Leica DMI4000B epifluorescence microscope provided by the Roskelley Lab (Life Sciences Institute, UBC, Vancouver, Canada). Image pseudocolouring and addition of scale bars were done with ImageJ (National Institutes of Health).

2.2.8 Statistics

A comparison of mean cell area (\( \mu \text{m} \)) was done using the paired, two-tailed Wilcoxon non-parametric test in GraphPad Prism 7 (GraphPad Software). The Wilcoxon matched-pairs signed rank test assumes samples are paired (e.g. two conditions were assessed using the same cell type) but does not rely on values being sampled from a Gaussian distribution (non-parametric). Significance can reflect changes that occur in either direction (i.e. higher or lower, hence two-tailed), and is based on the 95% confidence interval, with \( P<0.0001 \) denoting the highest statistical significance: (ns): not significant, \( P: 0.01 \) to 0.05): *, \( P: 0.001 \) to 0.01): **, \( P: 0.0001 \) to 0.001): ***, \( P<0.0001 \): ****.
Chapter 3: Results

3.1 The role of Cx43 in mechanical perturbations to J558µm3 cells

3.1.1 The expression of Cx43 does not influence the activation of the Rap1 GTPase by physical forces applied to cells.

3.1.1.1 Rationale

The Rap1 GTPase is a regulator of actin cytoskeletal dynamics in B cells (Lin et al., 2010). The expression of Cx43 in J558µm3 Cx43-negative cells resulted in increased and sustained BCR-induced Rap1 activation that was lost upon expression of a tail-truncated version of Cx43 (ΔCT246 Cx43) (Machtaler et al., 2011). In addition, the effect of Cx43 on BCR-mediated cell spreading was dependent on Rap1 activation (Falk et al., 2014a). The goal was to investigate the importance of the Cx43 CT on signaling reactions that regulate cytoskeletal rearrangements in B cells by characterizing BCR-induced Rap1 activation in transfected cell lines expressing a panel of CT truncation mutants (ΔCT246 Cx43, ΔCT258 Cx43, and ΔCT307 Cx43) and CT point mutants (constructed by May Dang-Lawson and Farnaz Pournia (Pournia, 2015)). The region of the Cx43 CT between aa 246-307 had previously been identified as important for supporting BCR-mediated cell spreading (Pournia, 2015). Therefore, I hypothesized that this domain of the Cx43 CT was also crucial in mediating BCR-induced Rap1 activation.

3.1.1.2 Rap1 in J558µm3 cells can be activated by factors other than receptor signaling

Rap1 activation assays were performed to compare J558µm3 Cx43-negative and Cx43+ cells that had undergone BCR stimulation (using anti-mouse IgM antibodies) as outlined in section 2.2.2.1. There were difficulties in obtaining consistent results that prevented the formation of definitive conclusions. This is illustrated in Figure 3.1A where the amounts of active Rap1 proteins after BCR stimulation from three independent experiments are shown. The control samples labeled with “0” were treated identically to the other samples except PBS was used instead of anti-IgM. It was clear over the course of three replicates that there was substantial variation in Rap1 activation. While in all
cases, the cells expressing wild type Cx43 always showed higher levels of active Rap1, or more sustained levels of Rap1 compared to the control cells, the variation of this assay would pose a problem as the assay could fail to detect subtle changes of mutated Cx43 versions compared to the wild type version of Cx43. I thus asked whether there were factors other than BCR stimulation that could be causing the lack of consistency in the activity of Rap1. To answer this question, cells were stimulated with PBS instead of anti-mouse IgM to see whether Rap1 would be activated regardless of BCR stimulation. Shown in Figure 3.1B are western blots probing for activated Rap1 in cells stimulated this way and were done within the same experiments as in figure 3.1A. PBS alone could induce Rap1 activation in the absence of BCR stimulation. Again, Rap1 activation varied across replicates even within the same cell type.
**Figure 3.1. Rap1 GTPase activation is influenced by mechanical disturbances.**

**A:** J558µm3 Cx43-negative and Cx43+ cells were stimulated with 20 µg/ml anti-mouse IgM antibodies for the indicated times. The ‘0’ control is stimulated similarly but with PBS and not anti-IgM. Lysates then went through a RalGDS-GST pulldown procedure before being probed for Rap1 during western blotting.

**B:** The exact same experiments as in (A) but here cells were stimulated with only PBS (denoted by having an asterisk beside the time point in blue font) for the indicated times.

Shown in **A** and **B** are three independent experiments with their accompanying loading controls underneath each row. Total Rap1 proteins were probed on a separate blot.

**C:** Mechanical disturbances prior to cell stimulation were assessed. N: the normal method of mixing cells in a flask, a combination of pipetting up and down with a 25 ml pipette. 5’: stimulation with 20 µg/ml anti-IgM for 5 min. H: flask is hit several times to mix the media. V: cells are vigorously pipetted up and down with a 25 ml pipette in the flask. Cells were stimulated and then lysed for pulldown for Rap1 (lane 1) or lysed immediately for pull down without stimulation (lane 2-4). Shown is representative data from three independent experiments.
It was possible that something in the manner in which the assay was performed could affect Rap1 activation. Since mechanical stimuli such as tension or shear stress have been shown to influence the activity of small GTPases like Rac (Katsumi et al., 2002) and more notably Rap1 (Freeman et al., 2016), I asked whether sample handling could be a factor that influences the activation of Rap1. In particular, cell preparation steps prior to stimulation or during stimulation when cells were mixed to ensure equal exposure to stimulating antibodies could be physically disturbing the cells. Therefore, different ways to mix cells as described in Figure 3.1C were compared. At this stage these different ways of handling the cells prior to stimulation did not cause substantial differences in Rap1 activation. Importantly, when cells did not go through a BCR stimulation process (lanes 2-4), there was only minimal amounts of active Rap1 as opposed to cells that did (lane 1) (Figure 3.1C). This suggested that perhaps the underlying factor occurs during cell stimulation.

3.1.1.3 Turbulence and shear forces can activate Rap1 GTPase in J558µm3 cells independent of Cx43 expression

Cell stimulation during a typical Rap1 activation assay involves the pipette addition of stimulants followed by inversion-induced turbulence afterwards to mix. Figure 3.2A demonstrates that even in the absence of BCR-crosslinking antibodies, disturbance by a pipette tip causes slight Rap1 activation that rises to almost the same extent as a BCR-stimulated sample when coupled with strong inversion-induced turbulence. It was now evident that mechanical force is an important underlying factor. Therefore, it was important to ask whether other forms of mechanical stress can induce Rap1 activation. Cells were passed back and forth through a 26G syringe needle (replacing BCR stimulation) to mimic the application of shear forces that lymphocytes experience during circulation in the bloodstream (Carey et al., 2011; Valignat et al., 2013). The result is shown in Figure 3.2B: compared to a sample that is left undisturbed by mechanical means, shear forces are able to activate Rap1 in J558µm3 cells. Finally, both Cx43-negative and Cx43+ cells experienced similar degrees of Rap1 activation in response to turbulence and shear forces (Figure 3.2C, D). In other words, cells within a liquid medium that is physically perturbed by repeated inversions
(turbulences) or passaged through a needle multiple times (shear) is sufficient to cause Rap1 activation in the absence of BCR signaling in J558µm3 cells regardless of Cx43 expression.

3.1.1.4 Turbulence and shear forces can activate Rap1 GTPase in other B cell lines

Mechanical force-induced Rap1 activation has been described in fibroblasts (Tamada et al., 2004), melanoma cells (Freeman et al., 2016), T cells and other hematopoietic cell lines (de Bruyn et al., 2003) but not in B cell lines. To further investigate whether this phenomenon was unique to J558µm3 terminally-differentiated plasmacytoma cells, force-mediated Rap1 activation in two other commonly used B cell lines in the lab (WEHI231, immature B cells; and A20, mature B cells) as well as primary bone marrow-derived mast cells were tested. Cells were mechanically stimulated as in the previous section and probed for active Rap1 using western blotting (section 2.2.5.1). Rap1 can be activated by physical stress in both WEHI231 and A20 B cells, with WEHI231 cells exhibiting greater Rap1 activation following mechanical stimulation than A20 cells (Figure 3.3A, B). However, this may be due to a higher expression level of Rap1 inherently in WEHI231 cells. Interestingly, Rap1 in mast cells did not appear to be as sensitive to turbulence forces at both the 5-minute and shorter 3-minute time points (Figure 3.3C). A 3-minute time point was also tested because prior experiments from members of the Gold lab had suggested that Rap1 activation in mast cells occur rapidly after FceRI stimulation.
Figure 3.2. Mechanical forces activate Rap1 GTPase in J558µm3 cells independent of Cx43 expression.

J558µm3 Cx43-negative and Cx43+ cells were stimulated as listed below:

A and B are experiments done with only Cx43-negative cells. C and D compares Cx43-negative and Cx43-expressing together to assess the effects of Cx43 expression on Rap1 activation induced by turbulence and shear, respectively.

A: 0'; unstimulated, undisturbed. P; pipetting motions mimicking addition of anti-mouse IgM. 3x; strong inversion three times. 5'; stimulation with 20 µg/ml anti-mouse IgM for 5 min.

B: -; unstimulated, undisturbed. +; passage through 26G syringe needle four times and then incubate in 37°C for 5 min.

C: 0'; unstimulated, undisturbed. 4x; strong inversion four times. 5'; stimulation with 20 µg/ml anti-mouse IgM for 5 min.

D: -; unstimulated, undisturbed. +; passage through 26G syringe needle four times and then incubate in 37°C for 5 min.

Cells were then lysed and lysates were put through the RalGDS-GST pulldown procedure before being probed for Rap1 during western blotting. Total Rap1 proteins were probed on a separate blot. Shown are representative data from three independent experiments.
Figure 3.3. Activation of Rap1 GTPase in B cell lines by mechanical forces.

WEHI231 and A20 cells were stimulated as listed below:

A: 4x; strong inversion four times. 5*; stimulation with PBS for 5 min. 5'; stimulation with 20 µg/ml anti-mouse IgM for 5 min.

B: -; unstimulated, undisturbed. +; passage through 26G syringe needle four times and then incubate in 37°C for 5 min.

IgE receptors (FcεRI) on mast cells had been incubated with 1 µg/ml of anti-DNP IgE overnight in culture prior to experiment:

C: 0'; unstimulated, undisturbed. 4x; strong inversion four times. 3* or 5*; stimulation with PBS for 3 or 5 min. 3' or 5'; stimulation with 100 ng/ml DNP-BSA for 3 or 5 min as controls.

In A, B, and C, cells were lysed and lysates were then put through the RalGDS-GST pulldown procedure before being probed for Rap1 during western blotting. Total Rap1 proteins were probed on a separate blot. Shown are representative data from two independent experiments.
3.1.1.5 Summary

The Rap1 GTPases can be activated by mechanical forces in J558µm3 and other B cell lines, and Cx43 expression did not influence this activation. However, this presented an obstacle for the original goal of using the Rap1 activation assay as a means to quickly characterize the signaling responses of cells expressing Cx43 CT truncations and point mutations, and correlating these signaling results with cell biological assays like BCR-induced cell spreading, or chemokine receptor induced motility and migration. The Rap1 activation assay would not be ideal unless these mutants displayed drastic differences in active Rap1 that inconsistencies introduced by sample handling could be ignored. Since J558µm3 already contained all Cx43 CT mutants, we decided to assay the CT’s effect by assessing alternative signaling proteins associated with the BCR signaling pathway with these cells (section 3.2).

3.1.2 Mechanical cell stretching of J558µm3 cells does not perturb the actin cytoskeleton organization and is not influenced by Cx43 expression

3.1.2.1 Rationale

Expression of Cx43 resulted in increased cell spreading on anti-mouse IgM (anti-BCR)-coated glass coverslips in response to BCR signaling in J558µm3 cells (Machtaler et al., 2011), a complex process that involves the rearrangement of the cellular cytoskeleton. It was unclear whether cytoskeletal changes, in particular actin rearrangement, could be induced by physical stretching of the cell membrane without receptor signaling. It was also unclear whether or not this would be affected by Cx43 expression. Therefore, we tested whether Cx43 had a role in cytoskeletal changes induced by mechanical membrane stretching in J558µm3 in the absence of receptor signaling. To do this, we induced membrane expansion without receptor signaling by performing cell stretching experiments. Membrane stretching is done by adhering cells onto a flexible silicone surface, that can be stretched, using vacuum pressure (section 2.2.5.2). Cells that are attached on the silicone thus expand their area as the membrane is stretched. There were three criteria that needed to be satisfied preceding the cell stretching experiments. First, a protein that will bind to B cells that can coat the silicone surface had
to be identified. Second, the coating protein should enable cell attachment to silicone without causing the activation of receptor signaling pathways. Third, this attachment must be firm enough to also allow cells to be stretched. The goal was to fulfil these criteria before performing cell stretching.

3.1.2.2 Anti-MHC I antibodies as a coating protein to support cell attachment on silicone

Fibronectin has been previously used to coat silicone for cell stretching (Freeman et al., 2016). However, this was not suitable for the purpose of studying the effects of mechanical stretch on cytoskeleton dynamics because fibronectin is a ligand that engages integrin receptors that can lead to Rap1 activation (Harburger and Calderwood, 2009; Lin et al., 2010). Alternatively, MHC class I and II are common surface proteins that were considered instead because when cross-linked, they have been shown before to not cause widespread tyrosine phosphorylation of proteins in B cells (Gold et al., 1990). Class I molecules are found ubiquitously on the surface of nucleated cells in vertebrates and function to present self-peptides to cytotoxic T lymphocytes. This essentially provides continuous advertisement of the cell’s contents to the immune system. In contrast, class II proteins are expressed by APCs, such as B cells that have encountered antigen. The antigen is internalized by the B cell, processed into peptides, and re-appears on the cell surface bound to MHC II proteins. Recognition of the MHC II-peptide complexes by helper T cells can send additional activation signals to the B cell (Janeway, 2001; Penn, 2002) (section 1.2.1). Members of the Gold lab had previously used anti-mouse MHC II antibody-coated glass coverslips to adhere A20 B cells (Wang et al., 2017). The goal was to assess whether antibodies against MHC class I or II proteins can be used to coat a silicone surface and mediate cell attachment.

J558µm3 cells were assessed by flow cytometry (section 2.2.4) to determine the expression of MHC class I and/or II proteins. Cells were stained with mouse anti-mouse MHC class I primary antibodies coupled to Alexa Fluor® 488-conjugated anti-mouse IgG secondary antibodies or with rat
anti-mouse MHC Class II FITC-conjugated antibodies alone. These experiments show that J558µm3 cells express abundant MHC I but not MHC II proteins (Figure 3.4A).
Figure 3.4. MHC class I proteins are expressed on J558µm3 cells.

A: J558µm3 and WEHI231 cells were prepared for flow cytometry analysis as outlined in section 2.2.4. Cells were stained using mouse anti-mouse MHC I primary antibodies and detected with goat anti-mouse IgG-Alexa Fluor® 488 secondary antibodies, or rat anti-mouse MHC Class II FITC-conjugated antibodies alone. Fluorescent intensities versus cell count were plotted using FlowJo® analysis software.

B: J558µm3 cells were stimulated with 3 µg/ml anti-mouse MHC I or 20 µg/ml anti-mouse IgM antibodies for the indicated times and lysates were probed for tyrosine-phosphorylated proteins using anti-phosphotyrosine (4G10) primary antibodies coupled to the anti-IgG kappa light chain-HRP protein for secondary detection (section 2.1.1.2). Actin loading controls were obtained by incubating blots with stripping buffer and re-applying anti-β-actin antibodies.
The next concern was whether crosslinking of MHC I proteins would induce intracellular signaling that alters the cellular cytoskeleton. Class I proteins are composed of two separate α and β polypeptide chains where only the α chain is transmembrane and contains a cytoplasmic domain of approximately 30 aa (Penn, 2002). Because phosphorylation of cellular targets in response to receptor signaling is one of the most common post-translation protein modifications and governs a wide range of biological processes (van der Laarse et al., 2018), database searches for putative phosphorylation sites on the α chain were performed. The online Eukaryotic Linear Motif bioinformatics resource (http://elm.eu.org/) predicted modifications by Casein kinase 1 (CK1), Glycogen synthase kinase 3 (GSK3), and Phosphatidylinositol 3-kinase-related kinases (PIKK) kinases. However, these proteins normally do not have significant roles in lymphocyte activation (Eide and Virshup, 2001; Sutherland and Cohen, 1994; Imseng et al., 2018). The online resource also identified endocytic and ubiquitination motifs but these were predicted to reside in extracellular portions of MHC I. In the literature, an increase in intracellular calcium, as well as tyrosine phosphorylation of proteins, such as the tyrosine kinases Syk and Lyn, are seen in both B and T cells after MHC I crosslinking (Gur et al., 1990; Skov, 1998). While another study has further shown that intracellular calcium increases correlated with MHC I expression levels on B and T cells (Pedersen et al., 1996), a direct association of MHC I with BCR / TCR signaling complexes have not been demonstrated.

Experimentally, it has been shown that A20 cells incubated on anti-mouse MHC II-coated coverslips do not undergo cellular membrane spreading in reponse to BCR stimulation to the same extent as they do when on anti-mouse IgG-coated coverslips (Wang et al., 2017). Additional assessment of the protein tyrosine phosphorylation profile of J558µm3 cells after soluble stimulation with anti-mouse MHC I and anti-mouse IgM antibodies found that phosphorylation signatures were much more pronounced after BCR stimulation in comparison to anti-MHC I crosslinking (Figure 3.4B). Therefore, we reasoned that using anti-mouse MHC I proteins to fasten cells onto silicone is feasible.
3.1.2.3 Anti-MHC I antibodies can coat silicone, be detected by secondary antibodies, and support J558µm3 cell attachment

The next step was to assess whether anti-mouse MHC I antibodies can coat silicone rubber and support J558µm3 cell attachment. Silicone pieces of roughly the same area as 12 mm coverslips were carved out and a concentration of 2.5 µg/cm² anti-mouse MHC I antibodies (same concentration of anti-mouse IgM antibody solutions used for coating glass coverslips during the cell spreading assays (section 2.2.6)) was chosen to coat the silicone for testing cell adherence initially. A range of antibody concentrations (0.16 – 3.3 µg/cm²) and cell numbers plated, using both silicone pieces and full-size wells, were assessed and it was found that even coating silicone at a concentration of 0.25 µg/cm² anti-mouse IgM was enough to support attachment of at least 3 million J558µm3 cells (Figure 3.5). A summary of these different tests are presented in Figure 3.6. Detection by anti-mouse IgG-Alexa Fluor® 488 secondary antibodies is a viable method to determine whether silicone rubber has been coated with mouse anti-MHC I antibodies. A concentration of 0.33 µg/cm² or 1.66 µg/cm² anti-mouse MHC I coats silicone equally well and both concentrations are able to sustain J558µm3 cell attachment, with the number of cells attached governed simply by how many cells were incubated onto silicone or the concentration of antibodies. The method to attach cells was now established. Cell stretching experiments were performed using anti-mouse MHC I antibodies at 0.33 µg/cm² with an amount of cells that would allow space for membrane expansion to occur (~1-2 million cells per well).
Figure 3.5. Anti-mouse MHC I supports J558µm3 cell attachment on silicone sheets. The indicated amount of J558µm3 Cx43-negative cells suspended in 2.5 ml of culture media were incubated for 15 min on silicone wells that had been coated with 0.25 µg/cm² of mouse anti-mouse MHC I antibodies or no protein at all (non-coated) overnight at 4°C. Cells were stained with Rhodamine phalloidin for F-actin (red) and then imaged at 20x objective on a Leica DMI4000B epifluorescence microscope. Images enhanced: Brightness +40%, Contrast -20%. Images were pseudocoloured and added scale bars with ImageJ.
Figure 3.6. Variable cell adherence onto silicone sheets by anti-MHC I antibodies.

A: 3.32 µg/cm² mouse anti-mouse MHC I-coated silicone overnight at 4°C, no fluorophore-conjugated secondary antibody added.
B: Silicone that is not coated with any proteins (non-coated) and stained with anti-mouse IgG-Alexa Fluor® 488-conjugated secondary antibody.
C-E: The indicated amount of J558µm3 Cx43-negative cells suspended in culture media were incubated for 15 min on silicone pieces that had been coated with either 0.33 µg/cm² or 1.66 µg/cm² of mouse anti-mouse MHC I antibodies overnight at 4°C and stained with goat anti-mouse IgG-Alexa Fluor® 488 and Rhodamine phalloidin. Red: Rhodamine phalloidin (F-actin). Green: goat anti-mouse IgG-Alexa Fluor® 488. Imaged at 20x objective on a Leica DMI4000B epifluorescence microscope. Images enhanced: Brightness +40%, Contrast -20%. Images were pseudocoloured and added scale bars with ImageJ.

Green: Goat anti-mouse IgG-Alexa Fluor® 488
Red: Rhodamine Phalloidin (Actin)
3.1.2.4 Equibiaxial stretch in the absence of receptor-signaling does not perturb the actin cytoskeleton in J558µm3 cells on α-MHC I-coated silicone, regardless of Cx43 expression

The third goal was to ensure the attached cells can be mechanically stretched. Preliminary stretching experiments were done to determine the conditions best suited to study the effects of cell stretching in J558µm3 cells (section 2.2.5.2). Based on previous cell stretching conditions for A20 B cells (Wang et al., 2017), a 15% equibiaxial stretch for 5 min was chosen as a starting point before further testing the effects of differing durations of stretch and Cx43 expression on the actin cytoskeleton organization after stretch. Roughly 1-2 million cells were used per sample in all stretching experiments. These initial experiments suggested that mechanically expanding J558µm3 cells did not perturb the actin cytoskeleton organization as determined by immunofluorescence imaging. Adjusting the duration of stretch or expressing Cx43 did not change this outcome (Figure 3.7A, B). However, it was noted that at a magnitude of 15%, cells appeared crinkled and smaller upon stretching. To confirm whether this is caused by stretching, magnitudes of 10%, 18%, and 26% were tested which suggested that stretch forces greater than 10% did indeed cause cells to shrink and become wrinkled at the periphery (Figure 3.7C). At these higher magnitudes, cell stretching may be causing cell death that could interfere with our observation of the actin cytoskeleton. Therefore, experiments from this point onwards maintained stretch at 10% for 7 min because this duration also produced the greatest increase in cell area upon stretch (Figure 3.7D). The 7-AAD fluorescent DNA intercalator dye, which cannot pass through intact membranes, was also used to ensure cells were not dying after stretch. Figure 3.8 shows representative immunofluorescence images from 3 independent experiments of J558µm3 Cx43-negative and Cx43+ cells stretched at 10% cell elongation for 7 min each. Alexa-Fluoro 488-Phalloidin was used to stain for F-actin (green) and 7-AAD/DNA complexes appear in red. In all experiments, stretching J558µm3 cells did not induce drastic changes to the actin cytoskeleton organization regardless of Cx43 expression (Figure 3.8A). Cell viability was unlikely to be substantially affected by stretch because dead cells can be clearly distinguished with 7-AAD fluorescence (Figure 3.8B). Although Cx43 expression had differential effects on cell area across the
three experiments, when quantified together, Cx43 did not appear to have an effect on cell area after stretch (Figure 3.8C).
Red: Rhodamine Phalloidin (Actin)
Figure 3.7. Varying magnitudes and durations of stretch causes no detectable change to the actin organization in J558µm3 cells.

A: J558µm3 Cx43-negative cells were stretched at 15% cell elongation for the indicated times.
B: Cx43-negative and Cx43+ cells were stretched at 15% cell elongation for 5 mins.
C: J558µm3 Cx43-negative cells were stretched at the indicated magnitudes for 5 min.

In A, B and C, approximately 1-2 million cells were incubated on silicone wells coated with 0.33 µg/cm² of anti-mouse MHC I antibodies overnight at 4°C. Red; Rhodamine phalloidin (F-actin). Imaged at 100x oil objective, near the cell:silicone interface, on a III-Zeiss spinning disk confocal microscope. Images shown are from one experiment. Images enhanced: Brightness +40%, Contrast -20%. Images were pseudocoloured and added scale bars with ImageJ.

D: Quantifications plotted using GraphPad Prism 7 of cell area (µm) for A, B, C are shown. Graphs show median plus 95% confidence interval. -; un-stretched. Tested for significance using the Wilcoxon matched-pairs signed rank test. (ns): not significant, (P: 0.01 to 0.05): *, (P: 0.001 to 0.01): **, (P: 0.0001-0.001): ***, (P< 0.0001): ****.
Green: Rhodamine Phalloidin (Actin)
Red: 7-AAD/DNA complexes
Figure 3.8. Mechanically expanding J558µm3 cell size causes no detectable change to actin cytoskeleton organization regardless of Cx43 expression.
A: J558µm3 Cx43-negative and Cx43+ cells were stretched at 10% cell elongation for 7 minutes. Approximately 1-2 million cells were incubated on silicone wells coated with 0.33 µg/cm² of anti-mouse MHC I antibodies overnight at 4°C. Green; Alexa Fluor™ 488-conjugated Phalloidin (F-actin). Imaged at 100x oil objective, at the cell:silicone interface, on a III-Zeiss spinning disk confocal microscope. Images shown are representative from three independent experiments. Images enhanced: Brightness +40%, Contrast -20%. 3D reconstructions were done by stacking together 15 - 20 image slices taken at 0.2 µm intervals using SlideBook 6. Images were pseudocoloured and added scale bars with ImageJ.
B: Sample immunofluorescent images of J558µm3 Cx43+ cells stretched at 10% cell elongation for 7 mins and assessed for cell viability. Cells were incubated on silicone wells coated with 0.33 µg/cm² of anti-mouse MHC I antibodies overnight at 4°C. Green; Alexa Fluor™ 488-conjugated Phalloidin (F-actin). Red; 7-AAD/DNA complexes. Imaged at the cell mid-sections. Images enhanced: Brightness +40%, Contrast -20%. Images were pseudocoloured and added scale bars with ImageJ.
C: Pooled quantifications of cell area from three independent experiments after J558µm3 Cx43-negative and Cx43+ cells were stretched at 10% cell elongation for 7 minutes. - ; unstretched. Shown in green are the medians plus 95% confidence intervals. Tested for significance using the Wilcoxon matched-pairs signed rank test. (ns): not significant, (P: 0.01 to 0.05): *, (P: 0.001 to 0.01): **, (P: 0.0001-0.001): ***, (P< 0.0001): ****.
3.1.2.5 Summary

The motivation for assessing mechanically-induced membrane expansion on J558µm3 cells was to examine whether Cx43 had a direct effect on rearrangement of the cytoskeleton that was independent of receptor signaling. Cx43 expression had limited effect on cell morphology, size, and actin cytoskeleton organization in J558µm3 after mechanical stretch in the absence of receptor signaling. Coupled to the fact that Cx43 expression also did not influence Rap1 activation in response to non-receptor stimulations (section 3.1.1), it is likely that Cx43 is involved in the BCR signaling pathway to promote cell spreading of J558µm3 cells on anti-IgM coated coverslips.
3.2 The effect of Cx43 CT expression on BCR signaling in J558µm3 plasmacytoma cells

3.2.1 Rationale

Initiation of the BCR signaling cascade can lead to cytoskeletal remodeling through activation of the Rap1 GTPases (McLeod et al., 1998; McLeod et al., 2004; Batista et al., 2007). The knockdown of Cx43 in murine WEHI231 B cells resulted in decreased phosphorylation of various signaling proteins associated with the BCR signaling pathway (Choi, 2012). Loss of the Cx43 CT domain also attenuated BCR-induced Rap1 activation (Machtaler et al., 2011). Therefore, the goal was to investigate the importance of the Cx43 CT on BCR signaling responses. Rap1 activation was intended as a screening assay to characterize the effects of a multitude of Cx43 CT truncation and point mutants on BCR signaling reactions that regulate cytoskeletal rearrangements in B cells. However, since Rap1 activation can be triggered by non-receptor signaling (section 3.1), the effect of Cx43 CT mutations on other constituents of the BCR signaling pathway (as gauged by their phosphorylation state) was considered. We hypothesized that the Cx43 CT, which has previously been found to be important for supporting BCR-mediated cell spreading (Pournia, 2015), would also be critical for promoting BCR signaling responses.

3.2.2 The Cx43 CT is important for protein tyrosine phosphorylation in response to BCR stimulation in J558µm3 cells

3.2.2.1 ΔCT246 and ΔCT258 Cx43, but not ΔCT307 Cx43 result in decreased protein tyrosine phosphorylation upon BCR stimulation

J558µm3 Cx43-negative and cells expressing wildtype Cx43 or Cx43 CT containing truncations at a specific amino acid (i.e. ΔCT246 Cx43) were stimulated with anti-mouse IgM antibodies in solution to stimulate signaling through the BCR over a time course (section 2.2.2). The phosphorylation of total cellular proteins was examined by western blotting analysis using an anti-phospho-tyrosine antibody (section 2.2.3). Since tyrosine phosphorylation of proteins (pTyr) occurs rapidly upon BCR stimulation and regulates the activity and/or function of several key proteins within
the signaling cascade (Gold et al., 1990; Campbell and Sefton, 1990; Kirkham et al., 1994), pTyr was monitored to assess global perturbations to the BCR pathway upon loss of the Cx43 CT. Crosslinking of B cell receptors resulted in a gradual increase in the density of tyrosine-phosphorylated proteins over time compared to a PBS-stimulated control in wildtype Cx43-expressing cells. The absence of Cx43 expression or a removal of the CT (ΔCT246 Cx43) both decreased pTyr in response to BCR stimulation that was most prominent with proteins in the molecular weight range between 75 – 48 kDa (Figure 3.9A, B). To uncover which regions of the CT are important for supporting pTyr of total proteins, transfected J558µm3 cells containing additional CT truncation mutants (ΔCT258 Cx43 and ΔCT307 Cx43) were compared to J558µm3 cells expressing wildtype Cx43 cells. These ΔCT mutants were designed to allow us to identify the importance of different regions of the Cx43 CT. For example, the area proximal to the start of the CT contain a master regulatory region that includes SH2 / SH3 binding protein interaction motifs, as well as numerous residues modified by the Src and MAP kinases (Solan and Lampe, 2014; Ambrosi et al., 2016). The latter half of the CT include other phosphorylation sites, for Casein Kinase 1 (CK1), Protein Kinase A (PKA), and Protein Kinase C (PKC), which are important for gap junctional communication (Leithe et al., 2018). Truncation of the Cx43 CT at aa 258 led to a similar decrease in total protein pTyr as ΔCT246 Cx43-expressing or Cx43-negative cells upon BCR stimulation (Figure 3.9C). The ΔCT307 Cx43 mutant displayed an initial delay in pTyr but increased to densities closer to wildtype Cx43 cells starting by the 5-minute time point (Figure 3.9D). The results are quantified in Figure 3.9E, and F. In (E), the sum of band densities per lane from all lanes of each blot were measured. The mean densities from all experiments for each cell type are plotted against time. In (F), individual band densities from the 15 min time point of each representative blot are plotted against the molecular weight. Overall, expression of Cx43 in J558µm3 promoted the BCR-induced tyrosine phosphorylation of proteins. This was counteracted when the CT of Cx43 was removed (ΔCT246 Cx43) or truncated at aa 258, while cells expressing ΔCT307 Cx43 had a delayed total protein pTyr that increased by the 5-minute time point.
These data suggested that the region between aa 246-307 of the Cx43 CT is most crucial for mediating the BCR-induced tyrosine phosphorylation of proteins.

3.2.2.2 S279A, S282A but not S255A, S262A mutations to the Cx43 CT result in decreased protein tyrosine phosphorylation upon BCR stimulation

The BCR-induced protein tyrosine phosphorylation assay was extended to examine five additional serine-to-alanine CT mutants (S255A, S262A, S279A, S282A, 4S Cx43). These serine residues are targets of phosphorylation by MAPKs (Fong et al., 2014; Warn-Cramer et al., 1998) that become activated upon BCR stimulation (Gold et al., 2000). Phosphorylation of these amino acids are known to down-regulate gap junction communication (Fong et al., 2014). However, our lab has shown that S255A and S262A mutations still supported BCR-mediated cell spreading whereas S279A and S282A mutants did not, suggesting that channel communication and cytoskeletal changes associated with cell spreading can be separated from each other, and could be regulated by distinct mechanisms. It was thus of interest to assess the pTyr profiles of these mutants following BCR stimulation. S255A and S262A Cx43 CT mutations did not influence pTyr after BCR stimulation and these mutants exhibited a similar or higher degree of protein tyrosine phosphorylation for all time points compared to the wildtype Cx43-expressing cells (Figure 3.10A, B, E, F). In contrast, S279A and S282A mutations resulted in near identical pTyr between themselves but are noticeably reduced when compared to a wildtype Cx43 control (Figure 3.10C, D, E, F). Interestingly, when all four serine residues were mutated to alanine (4S Cx43), the pTyr profile of these cells expressing this mutant following BCR signaling was similar to wildtype Cx43-expressing cells (Figure 3.11). These data suggest that within the region of aa 246-307 in the Cx43 CT, serines aa 279 and aa 282 are important in mediating BCR-induced protein tyrosine phosphorylation in J558µm3 cells.
3.2.2.3 Summary

BCR-induced protein tyrosine phosphorylation is a simple assay that was used to further analyze the effects of a variety of Cx43 CT mutants. Further validation may be needed to confirm whether this assay is a reliable and reproducible signaling assay that can be adopted to characterize other Cx43 CT mutants. However, the present data suggested that the region between aa 246-307 of the Cx43 CT, in particular the serines at aa 279 and aa 282, are important for promoting tyrosine phosphorylation of total cellular proteins in response to BCR signaling in J558µm3 cells.
Anti-mouse IgM

A

WT Cx43  Cx43-negative

Time (min) 0 1 3 5 15 30

M. W. (KDa) 245 105 63 48 35 30

α-Phospho-Tyr (4G10)

α-Actin

B

WT Cx43  ΔCT246 Cx43

Time (min) 0 1 3 5 15 30

M. W. (KDa) 245 105 63 48 35 30

α-Phospho-Tyr (4G10)

α-Actin

C

WT Cx43  ΔCT258 Cx43

Time (min) 0 1 3 5 15 30

M. W. (KDa) 245 105 63 48 35 30

α-Phospho-Tyr (4G10)

α-Actin

D

WT Cx43  ΔCT307 Cx43

Time (min) 0 1 3 5 15 30

M. W. (KDa) 245 105 63 48 35 30

α-Phospho-Tyr (4G10)

α-Actin

E

J558μm3 Cx43-negative vs. Cx43 vs ΔCT Cx43

Protein tyrosine phosphorylation

Relative band density vs. Time (min)

- Cx43
- Cx43 ΔCT246
- Cx43 ΔCT258
- Cx43 ΔCT307
- Cx43-negative
Figure 3.9. Cx43 expression is important for BCR-induced protein tyrosine phosphorylation. J558µm3 Cx43-negative (A), Cx43, ΔCT246 (B), ΔCT258 (C), and ΔCT307 Cx43 (D)-expressing cells were stimulated with 20 μg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis (section 2.2.3) using pan-species anti-phosphotyrosine (clone 4G10) and anti-β-actin antibodies. Actin loading controls were obtained by incubating blots with stripping buffer and re-applying anti-β-actin antibodies. Western blots shown are representative of three independent experiments. E-F: Quantifications of the amount of tyrosine-phosphorylated proteins upon BCR stimulation. In (E), the sum of band densities per lane from all lanes of each replicate was measured. The mean densities pooled from all experiments for each cell type are then plotted against time. Error bars: Standard error of the mean. In (F), the illustration on the left showcases density tracings for individual bands from the 15 min time point of each representative blot plotted against the molecular weight. On the right, tracings are offset to better compare each tracing to one another.
A) WT Cx43 vs. S255A Cx43

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B) WT Cx43 vs. S262A Cx43

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C) WT Cx43 vs. S279A Cx43

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D) WT Cx43 vs. S282A Cx43

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E) J558m3 Cx43 vs. S255, 262, 279, 282A Cx43
Protein tyrosine phosphorylation

Relative band density vs. Time (min)

- WT Cx43
- S255A Cx43
- S255A Cx43
- S262A Cx43
- S279A Cx43
- S282A Cx43
Figure 3.10. Serines 279 and 282 of the Cx43 CT is important for BCR-induced protein tyrosine phosphorylation.

J558µm3 Cx43, S255A (A), S262A (B), S279A (C), S282A-Cx43 (D)-expressing cells were stimulated with 20 μg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis using pan-species anti-phosphotyrosine (clone 4G10) and anti-β-actin antibodies. Actin loading controls were obtained by incubating blots with stripping buffer and re-applying anti-β-actin antibodies. Western blots shown are representative of six independent experiments. E-F: Quantifications of the amount of tyrosine-phosphorylated proteins upon BCR stimulation. In (E), the sum of band densities per lane from all lanes of each replicate was measured. The mean densities pooled from all experiments for each cell type are then plotted against time. Error bars: Standard error of the mean. In (F), the illustration on the left showcases density tracings for individual bands from the 15 min time point of each representative blot plotted against the molecular weight. On the right, tracings are offset to better compare each tracing to one another.
**A**

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<th>Time (min)</th>
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**Anti-mouse IgM**

- α-Phospho-Tyrosine (4G10)
- α-Actin

---

**B**

**J558m3 Cx43 vs. 4S Cx43**

Protein tyrosine phosphorylation

Relative band density vs. Time (min)

**C**

**J558m3 Cx43 vs 4S Cx43**

Protein tyrosine phosphorylation

15 min time point

Relative band density vs. M.W. (kDa)
Figure 3.11. The 4S Cx43 CT mutant does not affect BCR-induced protein tyrosine phosphorylation.

A: J558µm3 cells expressing wildtype Cx43 and Cx43 with four serine-to-alanine mutations at aa 255, 262, 279, 282 simultaneously were stimulated with 20 µg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis using pan-species anti-phosphotyrosine (clone 4G10) and anti-β-actin antibodies. Actin loading controls were obtained by incubating blots with stripping buffer and re-applying anti-β-actin antibodies. Western blots shown are representative of three independent experiments. B-C: Quantifications of the amount of tyrosine-phosphorylated proteins upon BCR stimulation. In (B), the sum of band densities per lane from all lanes of each replicate was measured. The mean densities pooled from all experiments for each cell type are then plotted against time. Error bars: Standard error of the mean. In (C), the illustration on the left showcases density tracings for individual bands from the 15 min time point of each representative blot plotted against the molecular weight. On the right, tracings are offset to better compare each tracing to one another.
3.2.3 The role of the Cx43 CT in specific BCR signaling reactions in J558µm3 cells

3.2.3.1 Rationale

The Cx43 CT was found to promote tyrosine phosphorylation of proteins upon BCR signaling. However, it was unclear if there were specific proteins within the BCR signaling pathway that have their tyrosine phosphorylation disrupted in the absence of the Cx43 CT. It was also of particular interest to study the role of the Cx43 CT on BCR-induced signaling proteins that regulate the cytoskeleton, in addition to BCR signaling constituents in general. Therefore, J558µm3 wildtype Cx43-expressing cells and ΔCT246 Cx43 cells were stimulated with anti-mouse IgM antibodies in solution over a time course and the phosphorylation of major BCR-activated signaling proteins (Figure 3.12) was examined by western blotting analysis. Phosphorylation was chosen because the activity of many proteins within the BCR-signaling pathway is governed by their phosphorylation state (Hobeika et al., 2015; Abraham et al., 2016).
Figure 3.12. BCR signaling proteins assessed in this study.
A simplified diagram of the BCR signaling pathway showing signaling proteins (beige coloured) tested in the comparisons of J558µm3-Cx43 cells versus ΔCT246 Cx43 cells. Proteins with a blue highlight are tyrosine-phosphorylated upon BCR signaling.
3.2.3.2 The Cx43 CT does not affect BCR-induced phosphorylation of Lyn, BTK, PLCγ2, ERK, and Cofilin proteins

Antigen-induced clustering of the BCR triggers auto-phosphorylation of the Lyn tyrosine kinases that are associated with the BCR Igα/Igβ signaling subunits. Activated Lyn rapidly phosphorylates the ITAMs on Igα/Igβ (CD79α/β) to create docking sites for downstream effectors, namely the Syk tyrosine kinase (Hobeika et al., 2015; Abraham et al., 2016). Syk phosphorylates and recruits BLNK to scaffold the assembly of the BCR microsignalosome. This microsignalosome consists of the Btk, PLCγ2, Vav, and Grb2, among others. Btk is required for the activation of PLCγ2. In turn, PLCγ2 enables activation of the actin-severing protein, Cofilin, through production of DAG and activation of Rap1. DAG also helps to initiate the MAPK cascade, which includes activation of the ERK near the end of the pathway. The action of these proteins regulate gene expression, cytoskeleton reorganization, cell proliferation, and differentiation (Zhong et al., 2014). When the role of Cx43 in these pathways was assessed, it was found that the loss of the Cx43 CT did not affect the BCR-induced phosphorylation of Lyn, Btk, PLCγ2, ERK, or Cofilin as their pattern of phosphorylation was the same compared to cells that expressed wildtype Cx43 over the time course examined (Figure 3.13A-E).

3.2.3.3 The Cx43 CT may have roles in BCR-induced phosphorylation of CD79α, Syk, HS1, Pyk2 proteins

The phosphorylation of two other upstream components of the BCR signaling pathway, CD79α and Syk, were assessed. CD79α makes up part of the BCR signaling subunit. Tyrosine phosphorylation of its ITAMs is necessary for initiating downstream signaling cascades after antigen binding to the B cell receptor. In J558µm3 cells expressing wildtype Cx43, BCR signaling triggers phosphorylation of CD79α (and presumably the CD79β subunit) beginning at the 3 min time point and continues until 15 min where it decreases thereafter. In ΔCT246 Cx43-expressing cells, CD79α is phosphorylated in a delayed manner compared to wildtype Cx43; and in one experimental replicate only minor phosphorylation was seen in the 15 min and 30 min time points in the ΔCT246 Cx43-
expressing cells (Figure 3.14A). Subsequent repeats did not yield the same patterns of phosphorylation. The second protein evaluated was Syk. Syk contributes to the formation of and directly phosphorylates components of the BCR signalosome assembly (Abraham et al., 2016). Three independent experiments are shown in Figure 3.14B. The results were inconsistent but it is noted that both phospho-Syk and total Syk levels were likely too low to be properly detected by the western blotting method used. For this reason, whether the Cx43 CT affects CD79α and Syk activity remain unclear.

A role for the Cx43 CT in regulating cytoskeletal-associated proteins that are associated with the BCR signaling pathway were of special interest. Two proteins were analyzed. The first was HS1, or hematopoietic lineage cell-specific protein 1. This protein is a hematopoietic-specific homolog of Cortactin, an actin-binding protein important in regulating actin dynamics (Ammer and Weed, 2008) as well as being reported to interact with Cx43 (Squecco et al., 2006; Vitale et al., 2009). HS1 activity is regulated by tyrosine phosphorylation by Syk and also contains an Arp2/3 branched-actin polymerization protein binding domain (Gomez et al., 2006). It was unclear whether a loss of the Cx43 CT had any effect on the activity of HS1 because the pattern of phosphorylation was not consistent across three independent experiments even within the wildtype Cx43-expressing cells. HS1 appears to be the most highly phosphorylated at 15 min and overall there is a higher degree of phospho-HS1 compared to ΔCT246 Cx43 cells in the first replicate (Figure 3.15A). However, subsequent experiments were not able to replicate this result. I next examined Cx43’s effect on protein-tyrosine kinase 2 (Pyk2). Pyk2 is a non-receptor tyrosine kinase that regulates cell adhesion, spreading, migration in many cell types. Pyk2 also play roles in BCR-induced B cell spreading and its phosphorylation on critical activating residues is dependent on Rap1 activation (Zheng et al., 1998; Mitra et al., 2005), for which the Cx43 CT was important for (Machtaler et al., 2011). Like Syk and HS1, the phosphorylation of Pyk2 upon BCR signaling in both wildtype Cx43 and ΔCT246 Cx43 cells appeared random and no trend could be discerned (Figure 3.15B). Thus, the relationship between the Cx43 CT, HS1, and Pyk2 phosphorylation is uncertain.
Figure 3.13. The Cx43 CT does not affect BCR-induced phosphorylation of Lyn, BTK, PLCγ2, ERK, and Cofilin proteins.

J558µm3 cells expressing wildtype Cx43 and ΔCT246 Cx43 were stimulated with 20 µg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis. A: Anti-phospho-Lyn and anti-Lyn antibodies were used to probe for Lyn activity. Shown is a representative blot from three experiments. B: Anti-phospho-Btk and anti-Btk antibodies were used to probe for Btk activity. Shown is the result of one experiment. C: Anti-phospho-PLCγ2 and anti-PLCγ2 antibodies were used to probe for PLCγ2 activity. Shown is the result of one experiment. D: Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were used to probe for ERK activity. Shown is a representative blot from three experiments. E: Anti-phospho-Cofilin and anti-Cofilin antibodies were used to probe for Cofilin activity. Shown is the result of one experiment. Respective total loading controls were probed on a separate blot.
Figure 3.14. Cx43 CT in BCR-induced phosphorylation of CD79α and Syk.
J558μm3 cells expressing wildtype Cx43 and ΔCT246 Cx43 were stimulated with 20 μg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis using anti-phospho-CD79α and anti-CD79α antibodies to probe for CD79α phosphorylation (A) and anti-phospho-Syk and anti-Syk antibodies to probe for Syk activity (B). Shown are all three independent experiments. Respective total loading controls were probed on a separate blot.
Figure 3.15. Cx43 CT in BCR-induced phosphorylation of HS1 and Pyk2.
J558µm3 cells expressing wildtype Cx43 and ΔCT246 Cx43 were stimulated with 20 µg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis using anti-phospho-HS1 and anti-HS1 antibodies to probe for HS1 phosphorylation (A) and anti-phospho-Pyk2 and anti-Pyk2 antibodies to probe for Pyk2 activity (B). Shown are all three independent experiments. Respective total loading controls were probed on a separate blot.
3.2.3.4 Loss of the Cx43 CT results in decreased phosphorylation of the Ezrin / Radixin / Moesin proteins.

The Cx43 CT was found to be necessary for promoting global tyrosine phosphorylation of proteins in response to BCR stimulation. Efforts to uncover the specific enzymes of the BCR signaling pathway that are perturbed had not been successful. However, these comparisons did reveal that the loss of the Cx43 CT disrupted the phosphorylation of the ERM proteins in a consistent and reproducible fashion. ERM proteins are situated near the cell membrane where they act as linkages between the plasma membrane and the underlying cortical actin network, thereby contributing to membrane remodeling. ERM proteins are also involved in the BCR signaling pathway: they are phosphorylated (and thus active) in basal resting B cells but become transiently dephosphorylated after BCR signaling. Activity is later restored through binding to phosphatidylinositol 4,5-bisphosphate (PIP2) phospholipids followed by phosphorylation on a regulatory threonine residue in the C-terminal domain (Gupta et al., 2006; Parameswaran et al., 2011; Pore and Gupta, 2015). When the phosphorylation of ERM proteins was evaluated in wildtype Cx43 and ΔCT246 Cx43-expressing J558µm3 cells that are stimulated with anti-mouse IgM antibodies in solution, it was found that ERM proteins are initially active (i.e. phosphorylated) but become dephosphorylated upon BCR stimulation in Cx43-expressing cells (Figure 3.16A, B). In comparison, cells that express ΔCT246 Cx43 or that do not express Cx43 both have lower amounts of active, phosphorylated ERM at the basal, unstimulated cell state (i.e. the zero time-point) as well as across all other time points (Figure 3.16A, B). The data quantified in Figure 3.16C also indicate that ERM reaches total dephosphorylation at the same time as wildtype Cx43 cells even though the mutant Cx43-expressing and Cx43-negative cells had less phosphorylated ERM initially. Because BCR signaling is influenced by the mobility of the B cell receptors which are in turn limited by the cortical actin network (Treanor et al., 2010), these results suggest that Cx43’s role in BCR-induced cytoskeletal events could be through regulation of ERM proteins by the Cx43 CT.
Figure 3.16. Deletion of the Cx43 CT decreases phosphorylation of the ERM proteins. J558μm3 cells expressing wildtype Cx43 and ΔCT246 Cx43 were stimulated with 20 μg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis using anti-phospho-ERM and anti-ERM antibodies to probe for ERM phosphorylation in A and B. Loading controls were obtained by incubating blots with stripping buffer and re-applying anti-ERM antibodies. Western blots shown are representative of three independent experiments. C: band densities per lane from all lanes of each replicate were measured. The mean densities pooled from all experiments for each cell type are then plotted against time. Error bars: Standard error of the mean.
3.2.3.5 Summary

The goal was to investigate the importance of the Cx43 CT on the activity of BCR signaling proteins, particularly those with relevance to cytoskeletal reorganization. Assessment of protein tyrosine phosphorylation upon BCR stimulation indicated that a loss of the Cx43 CT had global effects on BCR signaling responses. Whether the Cx43 CT was important for the phosphorylation of specific BCR signaling-associated proteins remains unclear, and additional proteins that have also been attempted are listed in the Appendices. In the end, the survey of the BCR signaling pathway successfully identified the phosphorylation states of ERM proteins as being consistently perturbed in the absence of the Cx43 CT in J558µm3 cells.
Chapter 4: Discussion

4.1 Summary of main findings

4.1.1 Chapter 3.1: The role of Cx43 in mechanical perturbations to J558μm3 cells

Signaling from the B cell receptor induces rapid actin cytoskeleton remodeling in B cells (Fleire et al., 2006; Tolar, 2017). To study the role of Cx43 on cytoskeleton reorganization in B cells, we characterized the effects of Cx43 on BCR signaling responses. Cx43 had previously been found to be important in supporting a BCR-mediated cell spreading response (Machtaler et al., 2011) that was dependent on the activation of Rap1 GTPases (Falk et al., 2014a). Because Rap1 is a major regulator of actin dynamics necessary to drive B cell spreading (Lin et al., 2010), we sought to assess Cx43’s role in B cell signaling reactions governing cytoskeleton-dependent processes in the context of Rap1. The activation of Rap1 was monitored in BCR-expressing J558μm3 cells that had been stimulated with anti-mouse IgM (anti-BCR) crosslinking antibodies over a time course. Active, GTP-bound Rap1 GTPases were isolated from cell lysates by a GST-RalGDS-mediated pull-down procedure (McLeod et al., 1998) and examined by western blot analyses. No definitive conclusions could be drawn from these experiments for a variety of reasons. Most importantly, we found that Rap1 can be activated by mechanical forces in the absence of receptor signaling. The phenomenon of mechanical forces inducing Rap activation was not unique to J558μm3 cells as it was observed in other commonly used B cell lines in the lab. Cx43 expression appeared to play no role in this force-triggered Rap1 activation because J558μm3 cells experienced a similar degree of Rap1 activation in response to mechanical stress regardless of whether Cx43 is expressed (section 3.1.1). To further assess if Cx43 had an effect on the cellular actin cytoskeleton upon mechanical expansion of cell size by stretch force rather than receptor signaling, membrane stretching experiments were performed using the Flexcell® FX-5000™ Tension System to physically enlarge the cell area. J558μm3 cells were bound to anti-mouse MHC I antibody-coated silicone surfaces that became expanded under vacuum pressure while cells were attached. Cell stretching in the absence of receptor signaling
increased cell:silicone contact area but did not lead to obvious, detectable changes to the actin cytoskeleton in J558µm3 cells, as determined by immunofluorescence imaging using rhodamine phalloidin to detect intracellular actin. Likewise, Cx43 expression appeared to have limited influence because its expression (or lack of) made little difference to cell morphology, size, and actin organization after mechanical stretch (section 3.1.2). These data suggested that Cx43 does not regulate cytoskeleton changes in the absence of receptor signaling, and reinforces the notion that the importance of Cx43 in BCR-mediated cell spreading was likely through enhancing BCR signaling, or perhaps through other receptors that lead to cytoskeletal rearrangements.

4.1.2 Chapter 3.2: The role of the Cx43 CT in the BCR signaling pathway in J558µm3 cells

Cx43 is documented to have an effect on the BCR signaling pathway. Knockdown of Cx43 in WEHI231 cells decreased phosphorylation of key signaling proteins associated with the BCR signaling cascade (Choi, 2012). Cx43, in particular the CT domain, is crucial for heightened and sustained BCR-induced Rap1 activation (Machtaler et al., 2011). This hinted that Cx43 is regulating Rap1 activation within the BCR signaling pathway and that assessing Rap1 could be a simple readout to investigate the importance of the Cx43 CT in Rap1-mediated cytoskeletal rearrangements. However, because Rap1 could be activated by non-receptor-based stimulations as well, a screen to assess the Cx43 CT’s role in regulating other BCR signaling proteins ensued, with the goal being to identify an alternative readout with which we can use to characterize the importance of the Cx43 CT on BCR-mediated cytoskeletal rearrangements. J558µm3 cells were BCR-stimulated with anti-mouse IgM crosslinking antibodies over a time course and lysates were examined by western blotting analyses. Expression of Cx43 increased tyrosine phosphorylation of proteins in response to BCR signaling compared to Cx43-negative cells, and deletion of the Cx43 CT (ΔCT246 Cx43) resulted in an overall decrease compared to wildtype Cx43-expressing cells, suggesting the importance of the CT in total protein pTyr. Additional Cx43 CT truncation mutants (ΔCT258 Cx43 and ΔCT307 Cx43) showed that the region of the CT most important for this decrease is between aa 246-307. While
mutating serines 255 and 262 to alanine (no longer able to be phosphorylated) did not affect cellular protein pTyr after BCR signaling, we found that serines 279 and 282 likely played a role in supporting protein pTyr because mutations of these amino acids to alanine attenuated protein pTyr. Interestingly, serine-to-alanine mutations at aa 279, 282 as well as 255, 262 simultaneously (4S) caused no effect to protein pTyr following BCR stimulation. Overall this data indicated that the Cx43 CT had global effects on the BCR signaling pathway. The next step was to determine whether the Cx43 CT specifically acted on individual BCR signaling proteins. We found that the Cx43 CT appeared to have no effects on the activity of some proteins (Lyn, BTK, PLCγ2, ERK, and Cofilin). For others (CD79α, Syk, HS1, and Pyk2), we were unable to determine if the Cx43 CT had an effect based on the methods used. However, Cx43 and its CT was required for the phosphorylation of ERM proteins. Phosphorylation directly regulates the activation of ERM proteins (Clucas and Valderrama, 2014). And since activated ERM tether the plasma membrane and peripheral actin cytoskeleton together via transmembrane proteins such as Cx43, one role for Cx43 in cytoskeleton reorganization of B cells may be through controlling the activity of ERM proteins (discussed more in section 4.2.2).

4.2 Discussion

4.2.1 Chapter 3.1: The role of Cx43 in mechanical perturbations to J558µm3 cells

The Rap GTPases play important roles in BCR-induced actin reorganization and B cell morphological changes (Lin et al., 2008). Rap activation is a common event in intracellular signaling pathways and can be activated by many factors including stimulation of the T cell receptor (Reedquist and Bos, 1998), stimulation by cytokines (M'Rabet et al., 1998), second messengers such as calcium ions (Franke et al., 1997), as well as guanine nucleotide exchange factors (Bos et al., 2001). In B lymphocytes, Rap1 can become active in response to antigen receptor signaling through activation of PLCγ2. PLCγ2 cleaves PIP2 into the second messengers IP3 and DAG (Abraham et al., 2016). IP3 leads to a release of calcium from intracellular stores while DAG binds to DAG-regulated guanine exchange factors, such as RasGRPs (Stone, 2011) to activate Rap1. Active Rap1 mediates actin
reorganization through regulating the actin-severing protein, coflin (Freeman et al., 2011) as well as binding upstream activators of the Arp2/3 complex to promote actin polymerization (Bos, 2005; Freeman et al., 2011).

Rap1 is important in integrin “inside-out” signaling whereby engagement of leukocyte receptors (such as chemokine receptors) transmits signals within the cell that leads to rapid activation of Rap GTPases (Hynes, 1992; Kinashi and Katagiri, 2005). GTP-bound Rap can then recruit the adaptor protein RAPL and RIAM/Talin complexes to the cytoplasmic tails of integrins (Katagiri et al., 2003; Han et al., 2006), which result in unfolding of the integrin extracellular domains that increase their affinity for ligand (Abram and Lowell, 2007). Adhesion receptors, like integrins, bind to ECM proteins (e.g. fibronectin) and are one of the main transmitters of mechanical force into the cell (Ross et al., 2013). Binding to ECM proteins clusters integrins, increasing its avidity (Ginsberg, 2014) and results in formation of focal adhesions (Sastry and Burridge, 2000). Focal adhesions are multi-protein complexes that link integrin receptors to the actin cytoskeleton, thereby transferring external forces directly to the cellular cytoskeleton. Note that although immune cells express integrins, they generally do not express focal adhesion complexes (Huse, 2017). These complexes are initiated principally by the recruitment of Talin to the β-subunits of integrins. Talin binds cytoplasmic domains of integrins via its N-terminal FERM domain and to F-actin via its C-terminal rod domain (Ross et al., 2013). Talin is also a platform to recruit other binding partners such as paxillin and vinculin (Puklin-Faucher and Sheetz, 2009). Vinculin head and tail domains bind to Talin and actin, respectively, and these interactions determines the tensile strength of focal adhesions (Galbraith et al., 2002; Grashoff et al., 2010). Paxillin and Talin recruit focal adhesion kinase (FAK), which can phosphorylate other focal adhesion proteins or associate with Src to further signal propagation (Mitra et al., 2005). Interestingly, vinculin binding sites within the Talin rod domain are normally buried until stretched open by force (del Rio et al., 2009). Pulling force must also be applied to the integrin-ligand contact site to achieve full integrin binding potential (Astrof et al., 2006; Friedland et al., 2008;
Work by Freeman et al. has shown that the ability for melanoma cells to sense tension and respond accordingly through cytoskeletal remodeling is dependent on Rap activation. Hence, integrins are one of the main transducers of force into the cell that requires the activation of Rap as tension sensors (Freeman et al., 2016).

Mechanical stimuli are widespread in the body and can stem from mechanical compressive forces (e.g. in the bones), stretch forces (e.g. in the lungs), or fluid shear forces (e.g. in the vasculature) (Pruitt et al., 2014). Turbulence is a term used by de Bruyn et al. to describe physical disturbances that is caused by tumbling tubes to mix samples in solution. It is a broad term representing mechanical stresses that likely include fluid shear stresses (de Bruyn et al., 2003). In biological systems, fluid shear stress is defined as the frictional force derived from the flow of a biological fluid over cells or tissues. In this context, the main source of fluid shear stress comes from lymphatic or blood flow within circulation (Li et al., 2014b). Shear stress has important implications for vessel wall endothelial cells. Lymphatic flow drives increased secretion of chemokines and expression of adhesion molecules on the endothelial cell surface (Cinamon et al., 2001; Miteva et al., 2010). Fluid shear stress stimulates the formation of actin stress fibres in arterial endothelial cells (Franke et al., 1984), which remodel their cytoskeleton to orient stress fibers in the direction of blood flow (Abiko et al., 2015; Hahn et al., 2011; Ando et al., 1987). Shear stress also impacts B lymphocyte processes. B cells experience low shear stress when they are positioned near the centre of a blood vessel. Within seconds of coming into contact with vessel wall endothelium, a spike in fluid shear stress is imparted onto the B cell (Coughlin et al., 2008). The magnitude of this force equates to those normally experienced by vessel wall endothelial cells (Makino et al., 2007). Increases in shear stress and interaction with the endothelium stimulates the B cell transition from a passive to an active state characterized by the appearance of cytoplasmic processes and projections in preparation for directed-migration (Ohashi et al., 1996). In particular, shear flow can guide B cell migration as it was
found that shear flow is a directional cue acting via integrin-ligand interactions to direct splenic marginal zone B cell migration (Tedford et al., 2017).

Passage through a syringe needle mimics application of fluid flow shear force to cells, with both the fluid viscosity and needle bore size modulating the strength of the force (Wahlberg et al., 2018). Both parameters are kept constant when passaging J558µm3 cells through syringe needles to ensure constant forces are applied across experiments. Needle shearing and mixing-induced turbulence were found to activate Rap1 GTPases rapidly (within minutes) in J558µm3 plasmacytoma B cells. This form of stimulation triggered Rap1 activation in WEHI231 and A20 B cell lines as well (Figure 3.3). Rap1 in WEHI231 appeared to be relatively more sensitive to turbulence than J558µm3 because the amount of active Rap1 far exceeds J558µm3 when lysates from both were compared with the same exposure on a single blot. This may reflect a difference in the developmental stages of these two cell types. WEHI231 cells represent an immature B cell phenotype while J558µm3 cells are terminally-differentiated plasma B cells. Indeed, it was noted that mature megakaryocytes were less sensitive to mix stress-induced Rap1 activation compared to more immature megakaryoblasts (de Bruyn et al., 2003). WEHI231 B cells may also contain more membrane reserves, as seen in their ability to spread to a greater area than J558µm3 cells (Machtaler et al., 2011). Membrane reserves may allow cells to more readily adapt to changes in membrane tension (Hamill and Martinac, 2001). Primary bone-marrow derived mast cells, in contrast, did not react to mechanical stimuli. Rap1 was not activated in respond to turbulence in these cells. An interesting explanation could be due to their inherent role in the immune system. Dermographic urticaria is a disease in which the skin reacts to touch by becoming inflammed. This reaction is caused by mast cells that release proinflammatory mediators upon slight physical pressure (Lawlor et al., 1989; Metz et al., 2011). It is possible that mast cells are normally insensitive to low degrees of mechanical forces to prevent unwanted Rap1 activation, and/or cause unnecessary inflammation. In the diseased state, mast cells may exist in a hypersensitive state that is more responsive to mechanical stress.
An ability to remodel the cytoskeleton in response to mechanical stress is likely a reflection of the morphological flexibility and immune function of B cells (Huse, 2017). For example, B cells must be able to become deformed while preventing rupture as they squeeze through tight interstitial spaces (de Bruyn et al., 2003). B cells also employ actomyosin-dependent pulling forces during antigen internalization (Natkanski et al., 2013). These forces provide a way to test the strength of BCR-antigen contacts, resulting in affinity or avidity-driven extraction and internalization of only BCR signaling clusters that are able to maintain contact amidst cytoskeletal forces (Tolar and Spillane, 2014). However, the exact mechanisms that relay a shear stimulus to activate Rap1 in J558µm3 cells is not completely understood. Rap1 activation by shear has been reported previously in T cells, megakaryocytes (de Bruyn et al., 2003), and B16F1 melanoma cells (Freeman et al., 2016). Other GTPases such as Rac and Ras in fibroblasts have also been found to be sensitive to stretch forces (Katsumi et al., 2002; Sawada et al., 2001). Recall that immune cell types generally do not express classical focal adhesion complexes (Huse, 2017). They do, however, form transient adhesive contacts that contain proteins typically found in focal adhesions, such as Talin, Vinculin, and Paxillin (Stanley et al., 2008; Monks et al., 1998; Saez de Guinoa et al., 2013; Jarvis et al., 1997). Therefore, Rap1 activation through force-sensing by focal-adhesion like contacts is possible (Tedford et al., 2017). Yet, it is unlikely because J558µm3 cells were washed prior to stimulation and shear or turbulence stimuli proceeded in HEPES buffer that is devoid of serum. Danielewski et al. and de Bruyn et al. performed turbulence-induced Rap1 activation experiments in megakaryocytes under serum-starved conditions and also recorded identical results as presented here (de Bruyn et al., 2003; Danielewski et al., 2005). For this reason, shear-induced Rap1 is probably not mediated by integrin signaling. A variety of inhibitors against known proteins involved in receptor signaling pathways that involve Rap1 activation (PLCγ, PKC, PI3K proteins, chelators of calcium ions) as well as actin cytoskeleton stabilizing (jasplakinolide) or dissociating drugs (cytochalasin D, latrunculin A and B) did not disrupt turbulence-induced Rap1 activation (de Bruyn et al., 2003). This suggested that force-
induced Rap1 activation occurred via alternative proteins that are also independent of cytoskeleton dynamics.

Cell stretching stimulates the phosphorylation of a cryptic tyrosine residue within p130Cas (Sawada et al., 2006; Tamada et al., 2004). Phosphorylated p130Cas then recruits the C3G (also known as RAPGEF1) guanine nucleotide exchange factor and the Crk adaptor protein as a complex. C3G activates Rap GTPases upon cell stretching in fibroblasts (Tamada et al., 2004). Notably, shear forces can also uncover this tyrosine residue within p130Cas to recruit Crk (Okuda et al., 1999). Since p130Cas is expressed in J558μm3 cells, it is possible that Rap1 is being activated via phosphorylated p130Cas and the C3G/Crk complexes upon needle shearing. Another method that could activate GTPases are through adhesion molecules. Studies have shown that shear forces are essential in activation of lymphocyte adhesion molecules, namely the selectins. Lymphocyte selectins bind glycoproteins on endothelial surfaces (Ley, 2003) to initiate leukocyte rolling prior to transmigration through vessel walls. Optimal selectin-dependent adhesion requires fluid shear forces (Finger et al., 1996; Lawrence et al., 1997). Interestingly, activation of L-selectin triggers activation of the Rac2 GTPase and Ras pathway via the Src family tyrosine kinase Lck (Brenner et al., 1996). Turbulence and shear forces could be activating selectins which lead to Rap1 activation. Finally, because mechanically-induced Rap1 activation is independent of the ability of the cytoskeleton to reorganize, perhaps the signal is directly relayed by membrane proteins that undergo conformational changes upon sensing physical stress. Talin is a prime example where its extension allows recruitment of kinases, phosphatases, and GTP exchange factors that can propagate a signal (Pruitt et al., 2014). Proteins that associate close to the cell surface, such as the bin/amphiphysin/rvs domain (BAR) membrane curvature proteins, may also be capable of sensing mechanical stress (Mim and Unger, 2012). In this way, forces are sensed and transformed into biochemical signals leading to Rap1 activation.
Tensile stresses regulate cellular processes. For instance, the pressure and pulsating nature of blood flow stretches and expands artery walls, which promotes vascular smooth muscle cell differentiation (Orr et al., 2010). Interstitial fluid pressure stretches lymphatic endothelial cells and stimulates integrin-dependent proliferation (Planas-Paz et al., 2012). Cells gauge the ECM stiffness by tension exerted intracellularly through integrin focal adhesions (DuFort et al., 2011), and this is especially relevant for cancer metastasis where stiffening of the ECM can promote tumour cell invasion (Levental et al., 2009; Lu et al., 2012). Mechanical stretching is used to study the effects of membrane expansion. Micropipettes, magnetic beads, and atomic force microscopes have been traditionally used to study cell stretching (Sniadecki et al., 2008; Nishimura et al., 2008; Harshad et al., 2016). Other commercial options include the Flexcell Tension Systems (Flexcell® International) that have been become more ubiquitously used due to its well-characterized strain profiles and adaptable straining patterns (Dhein et al., 2014; Higgins et al., 2013). It was unknown whether membrane stretching alone could induce changes to the actin cytoskeleton in J558µm3 cells and whether Cx43 expression influenced this. J558µm3 cells were adhered to silicone wells of BioFlex® culture plates using anti-mouse MHC I antibodies, as opposed to ECM components such as fibronectin, to avoid triggering cytoskeleton remodeling through integrin receptor signaling. Cell stretching did not cause perceivable changes to actin cytoskeleton organization in J558µm3 cells and Cx43 did not have a clear impact on this outcome. It is known that uniaxial stretch thickens actin stress fibers along the axis of stretch in fibroblasts and cells align to become parallel to the direction of the force (Hoffman et al., 2011). Likewise, endothelial cells re-orient stress fibers perpendicular to the axis of stretch in a Rho-dependent manner (Huveneers and Danen, 2009). It is difficult to analyze the actin structures of J558µm3 in this manner because B cells do not form stress fibers (Woodside et al., 2003). Therefore, another question that arises is whether cells were stretched.

It was clear that the cell stretching experiments were able to expand the cell:silicone contact area and increase cell size. However, it did not seem as though all cells were uniformly stretched.
because it was common to see cells in the stretched samples remaining the same size as ones in the un-stretched samples. Therefore, when three independent experiments were quantified together it did not become apparent that cell area changed. Theoretically, J558µm3 cells can expand to twice its original size if comparing its cell size in BCR-mediated spreading assays (Machtaler et al., 2011). This means that equibiaxial stretches of up to 30-40% are possible. The cell stretcher can only output a maximum of 26% biaxial stretch and we settled at 10% for 7 minutes because at higher percentages the cells appeared to be dying (Figure 3.7). Anti-mouse MHC I antibodies were noted to be cytotoxic on the manufacturer website but it is unlikely to be relevant in this case because cells were only incubated on antibodies for less than 30 minutes before fixing. It is possible to only analyze the cells that have been affected by stretching compared to un-stretched cells by employing positive indicators.

For example, actomyosin interactions drive cytoskeletal contractility and expansion in immune cells (Tolar, 2017; Murrell et al., 2015). As phosphorylation of the myosin light chains (MLC) can regulate actomyosin activity (Kaneko-Kawano et al., 2012), cells can be stained for phosphorylated MLC as an indication for cytoskeletal changes. Likewise, the activation of p130Cas and Rap1 proteins are also possible indicators of whether cells have responded to the stretching stimuli (Tamada et al., 2004; Freeman et al., 2016; Sawada et al., 2006). Other proteins that have been shown to become upregulated upon stretch, such as ERK (Li et al., 2015), can be used as well. Western blots to probe for phosphorylated p130Cas in response to 15% equibiaxial stretches in J558µm3 cells had been attempted. Although p130Cas is abundantly expressed in these cells, no phosphorylated p130Cas was detected even when the antibodies at the same concentration could detect adequate amounts of phosphorylated p130Cas in A20 cells on the same blot. Alternative methods to enrich for pp130Cas in J558µm3 become western blotting may be necessary. But because Pyk2 is also required for Cas tyrosine phosphorylation (Astier et al., 1997), and J558µm3 does not appear to express abundant Pyk2 either, it is likely not feasible to assess p130Cas after stretching in these cells. Rap1 may be a better protein of choice. Lastly, uniaxial stretches may instead uncover more effects on the actin cytoskeleton by stretch in J558µm3 cells.
Connexin43 expression did not affect turbulence and shear-induced Rap1 activation nor any effects that mechanical membrane stretching may have on the actin cytoskeleton in J558µm3 B cells. If Cx43 were to have an effect on mechanical force-induced Rap1 activation, we would expect that the amount of active Rap1 proteins as a result of force stimuli be different when Cx43 is either expressed or not expressed. Similarly, if Cx43 did have an effect on actin cytoskeleton organization, we might expect that a loss of Cx43 influences the integrity of peripheral actin, given Cx43’s potential interaction with ERM proteins, such that the actin network becomes disorganized and punctated around the cell. These were not observed. However, it is interesting to speculate that Cx43 may have an effect on the cell:substrate contact area depending on the stiffness of the surface because Figure 3.7D suggested that Cx43-expressing cells have a smaller contact area than Cx43-negative cells on silicone rubber. This is opposite to what was observed before indicating that Cx43 expression causes larger cell:surface contact areas on glass coverslips (Machtaler et al., 2011), a much stiffer substrate. Note that the coverslips were incubated with anti-BCR antibodies whereas the silicone were incubated with anti-MHC I antibodies. Nonetheless, it may be of interest to re-visit this possible effect by Cx43 in future studies.

Cx43’s role in mechanotransduction in B cells have not been widely discussed but is well-documented for tenocytes and osteocytes. Tendons are collagenous tissues continually subjected to mechanical loads that relay mechanical forces from muscle to bone to effect movement (Wall et al., 2016). Tensile stress localizes Cx43 proteins with actin in tenocytes and this localization increases with longer durations of strain (Wall et al., 2007). Tenocytes respond to mechanical stimulation by propagating calcium ion waves via Cx43 gap junctions in a coordinated fashion among interconnected cells (Banes et al., 1999). Likewise, bones are constantly under mechanical pressure and osteocytes adapt to these stimuli by adjusting bone formation and resorption (Plotkin et al., 2015). Fluid flow increases Cx43 phosphorylation and localization on the plasma membrane, and biaxial stretching promotes cell-to-cell gap junction coupling in dye transfer assays (Ziambaras et al.,
1998; Alford et al., 2003). It is possible that Cx43 gap junction channels may play a role in the ability of B cells to sense mechanical stress. However, at the density that B cells are plated onto silicone wells, it is possible to see that stretching does not cause obvious changes to the actin organization in cells that were either close enough to make cell-cell contacts or were not (Figure 3.8). Wall et al. noted that tensile strain could not increase the co-localization of Cx43 with actin in COS-7 fibroblast cells compared to tenocytes. They speculated that this lack of strain response could be because COS-7 cells have a less robust actin cytoskeleton that are not as mechanically sensitive as tenocytes, which readily transduce mechanical signals (Wall et al., 2007). Given their developmental stage as terminally-differentiated, immotile cells, the same might be true for J558µm3. Hence why it is unable to effectively convey any effects by Cx43 in response to mechanical stretch. Yet, J558µm3 Cx43-negative cells are wholly capable of reorganizing its actin cytoskeleton to effect a spreading response upon B cell receptor signaling when Cx43 is expressed (Machtaler et al., 2011). Therefore, this rather suggests that irrespective of functional gap junction communication, when receptor signaling (e.g. from the BCR) is absent, Cx43 has no effect on the organization of the actin cytoskeleton in B cells.
4.2.2 Chapter 3.2: The role of the Cx43 CT in the BCR signaling pathway in J558µm3 cells

4.2.2.1 The importance of the Cx43 CT in BCR-induced tyrosine phosphorylation of proteins

The Cx43 protein is phosphorylated upon BCR signaling (Machtaler et al., 2011) and its carboxyl tail region is the primary site of phosphorylation by a number of different kinases (Solan and Lampe, 2009). We therefore explored the effects of the Cx43 carboxyl tail domain on the B cell receptor signaling pathway. In addition to cells expressing Cx43 with a carboxyl tail deletion (ΔCT246 Cx43), mutants expressing Cx43 with partially truncated CTs (ΔCT258 Cx43 and ΔCT307) were examined. The ΔCT258 mutant retains serine 255, a known target of MAPKs (Fong et al., 2014; Warn-Cramer et al., 1998) that are activated in response to BCR signaling (Gold et al., 2000). ΔCT258 also retains tyrosine 247, a target of Src kinase (Lin et al., 2001). Src phosphorylation at tyrosine 265 (which is lost in ΔCT258) creates an SH2 binding site (Lin et al., 2001) that can lead to subsequent phosphorylation on tyrosine 247. Interestingly, both tyrosine 247 and 265 are targets of Tyrosine Kinase 2 (Tyk2) as well and phosphorylation at these sites by Tyk2 inhibits gap junction communication (Li et al., 2016). Truncation at ΔCT258 removes an SH3 binding motif situated within a proline-rich region at aa 274-284 of the Cx43 CT that is important in recruitment of Src (Lin et al., 2001) and other SH3-domain-containing kinases. These sites are present in ΔCT307 Cx43-expressing cells along with serines 262, 279, and 282, which are additional targets for MAPK (Fong et al., 2014; Warn-Cramer et al., 1998). However, truncation at aa 307 removes sites for distal binding partners such as 14-3-3 (Park et al., 2006; Park et al., 2007), ERM (Pidoux et al., 2014; Dukic et al., 2017a), and phosphorylation sites by CK1 (Cooper and Lampe, 2002), PKA (Atkinson et al., 1995), PKC (Reynhout et al., 1992) kinases that regulate gap junction communication (Leithe et al., 2018).

Tyrosine phosphorylation of proteins governs signal transduction in many cellular processes, including B cell activation (Gold et al., 2000; Campbell and Sefton, 1990; Kirkham et al., 1994). This
post-translational modification regulates protein activity and phosphotyrosine residues are recognized by other proteins containing SH2 domains. In doing so, tyrosine phosphorylation permits protein interactions and propagation of signaling pathways through recruitment of adaptor proteins or enzymes acting on downstream effectors (Hunter, 2009). The expression of Cx43 in J558μm3 cells increased total protein pTyr in response to BCR signaling, but loss of the Cx43 CT (ΔCT246 Cx43) reverted this effect and the protein pTyr profile in these cells became similar to those of Cx43-negative cells. Additional Cx43 CT truncation mutants (ΔCT258 Cx43 and ΔCT307 Cx43) revealed that the region between aa 246-307 is most important for the increase in protein pTyr (Figure 3.9). And while simultaneous serine-to-alanine mutations at aa 255, 262, 279, and 282 together (4S) did not affect tyrosine phosphorylation of cellular proteins in response to BCR signaling (Figure 3.11), mutants expressing individual S279A and S282A mutations reduced protein pTyr (Figure 3.10). This suggests that serines 279 and 282 are key residues involved in Cx43-induced protein pTyr, although they likely cannot fully account for this effect because truncation at aa 307 still attenuated protein pTyr at early time points even though serines 279 and 282 are present. It is unknown why mutations at S255 and S262 reverts the decrease in pTyr, but suggests S255 and S262 may normally have antagonizing effects on S279 and S282. Note that Cx43 proteins exist as hexameric arrays on the cell surface and multiple Cx43 CT exist in close proximity to each other. Phosphorylation of the CT is known to cause conformational changes in intrinsically disordered proteins such as the Cx43 CT domain (Spagnol et al., 2016a). S255 and S262 may have roles in determining the structural conformation of the CT, which can impact neighboring CTs as well. Structural analysis of Cx43 is beyond the scope of this thesis but the fact that mutations to S255 and S262 also affected total protein pTyr suggests they are residues of further interest.

The long carboxyl tail of Cx43 contains numerous protein interaction and phosphorylation sites, positioning it as a potential signaling scaffold for regulatory kinases and phosphatases (Matsuuchi and Naus, 2013; Sorgen et al., 2018). The first half of the Cx43 CT is a master regulatory
region (aa 246-296) that contains various critical phosphorylation sites and overlapping sequence motifs that form SH2 and SH3 binding domains to recruit kinases such as Src or proteins involved in Cx43 degradation such as NEDD4 (Ambrosi et al., 2016). Serines 279 and 282 are situated within the proline-rich region of aa 274-284 (PTAPLSPMSPP), which contains minimal consensus motifs (PxxP, where P is proline and x is any amino acid) for SH3 domain binding. This stretch of amino acids makes up the SH3-binding domain of the Cx43 CT and it is hypothesized that S279 and S282 regulate SH3 domain binding (Iyyathurai et al., 2018; Saksela and Permi, 2012; Zhou et al., 1999). Src tyrosine kinase interacts with the Cx43 CT SH3-binding domain to inhibit gap junctional intercellular communication through phosphorylation of Y246 and Y265 (Zhou et al., 1999; Giepmans et al., 2001). Src-family kinases, namely Lyn, Fyn, Blk, or Lck, are extensively involved in BCR signal transduction (Kurosaki et al., 1994; Gauld and Cambier, 2004). The ABL-family of non-receptor tyrosine kinases also contain SH3-binding capacity. ABL tyrosine kinases are expressed in B cells and have been reported to associate and phosphorylate the BCR coreceptor CD19 (Zipfel et al., 2000). Vertebrates encode two closely related paralogs, ABL1 and ABL2. Both are conserved through an SH3-SH2-TK (Src homology 3–Src homology 2–tyrosine kinase) domain cassette. While ABL1 includes nuclear localization signals and a DNA binding domain, ABL2 differs by having additional actin-binding ability (Colicelli, 2010). Indeed, ABL kinases are thought to play a role in cytoskeleton remodeling. The Mical redox enzymes were shown to oxidize actin filaments to mediated cofilin-driven actin disassembly (Grintsevich et al., 2016). It was later demonstrated that Mical synergistically activates ABL kinases through binding to its SH3 domain. Activated ABL in turn phosphorylates Mical on tyrosine 500 to enhance its redox activity (Yoon et al., 2017). Tyrosine 265 forms a tyrosine-based sorting motif (YxxO, where Y is tyrosine, X is any amino acid, and O is a bulky hydrophobic amino acid) enables an interaction with the adaptor protein complex AP-2 to mediate clathrin-dependent internalization of Cx43. Phosphorylated Y265 impedes AP-2 binding (Li et al., 2016). Therefore, in the case of ΔCT246 Cx43 and ΔCT258 Cx43 mutants, the loss of this phosphorylated tyrosine may impact the balance of Cx43 turnover. If the net effect is increased Cx43
internalization, then the recruitment of tyrosine kinases by Cx43 may be disrupted. Indeed, tyrosine-to-phenylalanine mutations at Y265 did appear to cause slightly decreased membrane localization of Cx43 as shown by EGFP fluorescence intensity compared to wildtype Cx43-expressing cells (Pourinia, 2015). Finally, the decrease in total protein pTyr upon mutation of S279 and S282 may also be due to over-recruitment of phosphatases. NEDD4, an E3 ubiquitin ligase involved in Cx43 degradation, binds the Cx43 CT via PY motifs (xPPxY, where P is proline, x is any amino acid, and Y is tyrosine) within the region aa 279-286 (SPMSPPGY) (Thomas et al., 2003; Leykauf et al., 2006). This interaction is regulated by S279 and S282 and phosphorylation on these serines increases the binding affinity of NEDD4 to the Cx43 CT by two-fold (Spagnol et al., 2016b). If this regulation is disrupted, Cx43 turnover could again be impeded and allow phosphatases that are recruited by Cx43 to continue dephosphorylating their target proteins for longer than usual. Relatively little is known about Cx43 and phosphatase interactions (Leithe et al., 2018). SHP-1 and SHP-2 tyrosine phosphatases modulate B cell signal transduction (Tamir et al., 2000) and may be potential candidate phosphatases. Another candidate is the T cell protein tyrosine phosphatase (TC-PTP). This protein dephosphorylates Cx43 at Tyrosine 247 and 265 and is found to counteract the role of Src in gap junction inhibition (Li et al., 2014a).

The CT region beyond aa 307 also appeared to be important in regulating total protein pTyr in response to BCR signaling, but the impact by this region on protein pTyr is likely small because ΔCT307 Cx43-expressing J558µm3 cells are able to induce similar pTyr at later time points whereas ΔCT246 and ΔCT258 mutants could not. Nonetheless, it is known that phosphorylation of S373 by Akt enables the binding of 14-3-3 proteins to a consensus 14-3-3 binding motif at aa 370-376 (RASSRPR) (Park et al., 2006). Interestingly, this binding motif is absent in other connexin proteins, indicating that 14-3-3 may have Cx43-specific functions (Leithe et al., 2018). Loss of phosphorylatable S373 by alanine substitution reduces 14-3-3 binding and concurrently reduces Cx43 degradation rate (Smyth et al., 2014), thereby affecting Cx43 turnover. Furthermore, 14-3-3 proteins
are abundantly expressed cytosolic proteins that participate in diverse signaling pathways through binding to a multitude of kinases and phosphatases. In this way, 14-3-3 is an important signaling regulator (Fu et al., 2000) that could help explain the small decrease in protein pTyr in ΔCT307 Cx43-expressing cells upon BCR signaling. An overall summary of the possible effectors that may be recruited by the Cx43 CT to modulate protein tyrosine phosphorylation after BCR signaling is presented in Figure 4.1.
**Figure 4.1. Protein interaction and phosphorylation sites on the Cx43 CT.**

A linear depiction of the rat Cx43 CT showing the effector proteins mentioned in this thesis that may be recruited by the Cx43 CT to potentially induce tyrosine phosphorylation of downstream targets upon BCR signaling. Phosphorylation sites that are targets of some of these proteins (see main text), as well as those with known functions from literature are also shown. The region and amino acids highlighted in red are found to be most important for Cx43-induced tyrosine phosphorylation of proteins following BCR signaling. PM: Plasma membrane; EC: Extracellular; IC: Intracellular; Src: Src kinase; Tyk2: Tyrosine kinase 2; TC-PTP: T cell protein tyrosine phosphatase; MAPK: Mitogen-activated protein kinase; CK1: Casein Kinase 1; PKA: Protein Kinase A; PKC: Protein Kinase C; AP-2: Adaptor protein 2; YxxO: Y, tyrosine, x, any amino acid, O, bulky hydrophobic amino acid; PP1: Protein phosphatase 1; NEDD4: Neural precursor cell-expressed developmentally downregulated gene 4; ABL: Abelson family non-receptor tyrosine kinases; SLK: STE20-Like Kinase; ERM: Ezrin/Radixin/Moesin; S: serine; P: proline. This figure is made with help from Lampe and Lau. *Int J Biochem Cell Biol.* 2004 and Leithe et al. *Biochim Biophys Acta.* 2018.
A decrease in the total amount of proteins that are tyrosine phosphorylated following BCR ligation suggested that the Cx43 CT had global effects on the BCR signaling pathway. However, it was unclear if specific proteins associated with this pathway were affected. BCR stimulation of J558μm3 cells with crosslinking anti-IgM antibodies followed by western blotting analysis with antibodies against specific signaling proteins of the BCR pathway revealed that a loss of the Cx43 CT had no effects on Lyn, BTK, PLCγ2, ERK, and Cofilin proteins (Figure 3.13). Its effect on CD79α, Syk, HS1, and Pyk2 phosphorylation was inconclusive (Figure 3.14 & 3.15). The greatest difference in total protein pTyr was within the molecular weight range between 75-35 kDa. It is possible that differences seen in the band at roughly 70 kDa could be attributed to Cx43-EGFP that is tyrosine phosphorylated, but the fact that these bands are present even in Cx43-negative cells indicate otherwise. It was especially puzzling to see that ΔCT246 Cx43-expressing cells did not have any difference in Lyn and Btk phosphorylation given that they are tyrosine phosphorylated upon BCR signaling (Abraham et al., 2016) and are within the correct weight range (56kDa and 73kDa, respectively). Syk (72 kDa) and BLNK (63-68 kDa) were additional tyrosine-phosphorylated proteins that could be affected by the loss of the Cx43 CT. However, inadequate amounts of Syk were detected by the method used and BLNK had only been assessed once and would need to be repeated before any conclusions can be made. Cofilin is normally activated by soluble anti-IgM stimulation and this is indicated by a characteristic de-phosphorylation within 5-10 minutes from a phosphorylated basal state in B cells (Freeman et al., 2011). The cofilin phosphorylation pattern seen in J558μm3 was the opposite and instead increased over time in response to stimulation. There are several explanations for these results. It is possible that the time course and stimulation dosage chosen was inadequate. Differences may exist (e.g. for Btk or Cofilin), but are too transient or occur at much later time points. Likewise, stimulation with 20 μg/ml of anti-IgM antibodies is a saturating concentration that may compensate for the ΔCT246 Cx43 mutant’s inability to respond, making it seem like there is no signaling defect in the mutant. These proteins would warrant revisits. For proteins that have ambiguous phosphorylation patterns, (e.g. CD79α and Syk), the amount of protein
that gets phosphorylated may be too low to be detected in whole-cell lysates. In this case, using immunoprecipitation to first enrich the protein of interest prior to using pan phospho-antibodies on the immunoprecipitates could permit better detection. If the phospho-fraction of the protein is indeed very low, sample handling steps could easily have caused inconsistencies, which reinforces the necessity for repeated validation. Subtle differences during blot development (antibody specificity, chemiluminescent reagent strength) could also be factors that impact the final blot outcome. Finally, perhaps the bands at the molecular weight range between 75-35 kDa in the total protein tyrosine phosphorylation blots do not necessarily belong to the BCR signaling pathway at all. Probing for specific BCR signaling-associated proteins to look for a similar trend seen in the tyrosine phosphorylations blots may not be searching in the right direction in that case.

4.2.2.2 The importance of the Cx43 CT in ERM phosphorylation and receptor clustering

Loss of the Cx43 CT consistently altered the phosphorylation of ERM proteins upon BCR signaling in J558µm3 cells. ΔCT246 Cx43-expressing and Cx43-negative cells both exhibited lower amounts of phosphorylated ERM at the basal, unstimulated state compared to cells expressing wildtype Cx43. The Cx43 CT could be recruiting proteins that are involved in the regulation of ERM activity. Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that has been shown to de-phosphorylate ERM proteins independent of PIP2 hydrolysis (Canals et al., 2012). Motif analysis of PP1 suggests that it may be able recruited to Cx43 through its SH2 domain, and this interaction could be lost when the Cx43 CT is removed. But because it is the phosphorylation of ERM that is affected, and ERM in all cell types were able to become fully de-phosphorylated by the 15-minute time point, it is more likely that Cx43 CT is recruiting ERM kinases rather than phosphatases. STE20-Like Kinase (SLK) is a serine/threonine kinase that is ubiquitously expressed in adult tissues and cell lines (Quizi et al., 2013). Its N-terminal kinase domain and carboxyl terminus share a high degree of homology with lymphocyte oriented kinase (LOK), a protein that have been reported to activate ERM proteins by phosphorylation (Belkina et al., 2009). The central coiled-coil domain of SLK may allow
an SH3 domain-mediated interaction with the Cx43 CT (Baron et al., 2015). SLK directly phosphorylates and activates mammalian ERM proteins (Machicoane et al., 2014) in addition to acting as a negative regulator of RhoA GTPases by phosphorylation on serine 188 (Al-Zahrani et al., 2013). Since Rho GTPases are important in B cell migration and lamellopodia formation (Tybulewicz and Henderson, 2009), this suggests an alternative possibility for how Cx43 can moderate cytoskeletal remodeling in B cells.

Dysregulation of ERM activity in the absence of the Cx43 CT offers another potential explanation for the decrease in total protein pTyr in response to BCR signaling. Transmembrane proteins do not freely diffuse throughout a uniform sea of lipids. An underlying actin cytoskeleton restricts the lateral diffusion of lipids (Fujiwara et al., 2002) and membrane-bound proteins such as the BCR (Treanor et al., 2010). Transmembrane proteins that possess the ability to connect with the cytoskeleton (e.g. Cx43) can act like “pickets” and are coupled to the sub-membrane actin via actin-linkage proteins such as ERM (Figure 4.2A). The interlinking actin network create “fences” that segregate and limit the diffusion of membrane proteins (Kusumi et al., 2005; Klammt and Lillemeier, 2012). Therefore, peripheral actin regulates BCR signaling by limiting BCR diffusion and experimental evidence has demonstrated that increasing BCR mobility through breakage of actin barriers alone is sufficient to cause robust BCR signaling without antigen stimulation (Batista et al., 2010; Treanor et al., 2010; Mattila et al., 2013). ERM proteins are known to undergo rapid and transient dephosphorylation upon BCR stimulation (Figure 4.2B). ERM then adopts a closed conformation that is incapable of binding to actin (Treanor et al., 2011). This enables enhanced BCR mobility and facilitates coalescence of BCRs with CD19 and lipid raft residents such as Syk and Lyn to phosphorylate ITAM motifs on CD79α/β and recruit downstream signaling proteins, forming BCR microsignalosomes (Gupta and DeFranco, 2003; Gupta et al., 2006; Harwood and Batista, 2010) (Figure 4.2C). ERM proteins are eventually re-phosphorylated upon continuous antigen stimulation. This re-establishes actin barriers that help the continued clustering of BCR microsignalosomes with
activating co-receptors (Treanor et al., 2011; Pore and Gupta, 2015) (Figure 4.2D). The number of signaling clusters formed determines whether BCR signaling will exceed the threshold for B cell activation (Harwood and Batista, 2010; Treanor et al., 2010; Fleire et al., 2006; Abraham et al., 2016). If the Cx43 CT is normally involved in ERM regulation, it could directly influence BCR signaling by regulating receptor clustering (Figure 4.2B, C). Additionally, Dukic et al. identified that a direct protein interaction exists between Ezrin and Cx43 in liver epithelial cells using immunofluorescence microscopy and proximity ligation techniques (Dukic et al., 2017a). The region of aa 366-370 (RASSR) in the Cx43 CT was critical for this interaction (Dukic et al., 2017b) and it is thought that Ezrin, together with zonula occludens-1 (ZO-1) anchors PKA in close proximity to the Cx43 CT to enable its phosphorylation by PKA (Dukic et al., 2017a). Cx43 may contribute to actin barrier formation by tethering the plasma membrane to cortical actin via direct Ezrin binding to promote cluster stability (Figure 4.2D). Not all lymphoid cell lines express Cx43, however, and ERM proteins have been suggested to bind to the ERM-binding motifs of CD43, CD44, and ICAM1 / ICAM2 (Yonemura et al., 1998; Tsukita et al., 1994). Interestingly though, the basal amount of phosphorylated ERM in Ramos B cells, a Cx43-negative cell line, is also lower compared to Cx43+ splenic B cells (Gupta et al., 2006). In the absence of the Cx43 CT, phosphorylation of ERM is decreased and Cx43-ERM binding is lost (Figure 4.3A). BCR signaling is able to cause ERM de-phosphorylation and the subsequent increase in BCR mobility (Figure 4.3B, C). However, overall BCR signaling (and thus protein pTyr) is attenuated due to an inability to reinstate actin barriers to promote BCR clustering and maintain microsignalosome integrity (Figure 4.3D). This offers a possible explanation for why there is a global decrease in protein tyrosine phosphorylation in ΔCT246 Cx43 or Cx43-negative cells compared to wildtype Cx43-expressing cells. A combined summary comparing the effects of Cx43 CT presence and absence on ERM phosphorylation, receptor clustering, and BCR clustering shown is shown in Figure 4.4.
Figure 4.2. Cx43 expression increases ERM phosphorylation and promotes BCR clustering. A simplified model of how Cx43 may be involved in the regulation of ERM phosphorylation and BCR clustering. A: During a basal resting state B cell receptors exists as small clusters on the cell surface. Their mobility is limited by the underlying actin cytoskeleton, which is linked to the plasma membrane through binding of ERM proteins to transmembrane proteins such as Cx43 or “Protein X”, which represents other ERM binding partners. B: BCR crosslinking by antigen triggers uncoupling of tethered ERM proteins. This uncoupling is dependent on ERM phosphatases (e.g. protein phosphatase 1) that may be recruited to the Cx43 CT. C: Ezrin uncoupling leads to a temporary increase in receptor mobility and BCR coalescence with co-receptors CD19 and lipid raft-associated BCR signaling proteins to form microsignalosomes. Signaling from BCR microsignalosomes induces Cx43 phosphorylation which take part in actin remodeling through promoting activation of Rap1 GTPases. Cx43 phosphorylation may help the Cx43 CT recruit ERM kinases (e.g. SLK) to phosphorylate inactive ERM proteins. D: This regulation, and/or the direct binding of ERM to Cx43 allows reformation of actin “corrals” which encourage continued aggregation of BCR microsignalosomes with signaling constituents to induce robust BCR signaling and tyrosine phosphorylation of downstream signaling proteins. The phosphorylated Cx43 CT may also be involved in recruiting kinases responsible for inducing protein tyrosine phosphorylation.
Figure 4.3. Deletion of the Cx43 CT reduces ERM phosphorylation and diminishes BCR clustering.

A simplified model of how the loss of Cx43 may influence the regulation of ERM phosphorylation and BCR clustering. 

A: Absence of regulation by the Cx43 CT results in less phosphorylated ERM at the basal resting state and abolishes a potential Cx43-ERM interaction. Phosphorylated ERM may still be binding to other transmembrane protein partners (e.g. Protein X).

B-C: The regulation of ERM de-phosphorylation and subsequent re-phosphorylation after BCR stimulation is altered due to the lack of ERM regulators that are normally recruited by the phosphorylated Cx43 CT.

D: The actin cytoskeleton undergoes remodeling and ERM may still be regulated by other factors that allow it to be re-phosphorylated to bind other protein partners. However, diminished regulation of ERM and/or loss of ERM binding in the absence of the Cx43 CT leads to attenuated receptor clustering due to a decreased ability to reinstate actin barriers. Relative to the situation in which wildtype Cx43 is present, B cell receptors do not interact long enough with CD19 or lipid raft-associated BCR signaling proteins to form lasting microsignalosomes. Ultimately, this is reflected by an overall reduction in BCR signalling and tyrosine phosphorylation of proteins. Decreased kinase recruitment in the absence of the Cx43 CT could also be contributing to attenuated BCR-mediated tyrosine phosphorylation of proteins.
Figure 4.4. Cx43 in regulation of ERM phosphorylation and BCR clustering.
A comparison of the effects of the Cx43 CT on ERM phosphorylation, receptor clustering, and BCR clustering. This figure is a combination of Figures 4.2 and 4.3. In A-D: the expression of Cx43 with an intact CT leads to increased ERM re-phosphorylation following BCR signaling and re-establishment of actin barriers by ERM leads to stabilized BCR clustering, signaling, and protein tyrosine phosphorylation. In E-F: deletion of the Cx43 CT abrogates Cx43-mediated regulation of ERM phosphorylation and activity. This in addition to a loss of potential ERM-Cx43 binding leads to decreased BCR cluster stability and thus an overall decrease in BCR signaling.
4.2.3 Implications

Putting the findings of this thesis into the context of what our lab has previously shown, Table 4-1 compares several Cx43 CT mutants to show their effects on the tyrosine phosphorylation of proteins upon BCR signaling, whether they contain the putative ERM binding site, and whether they enabled BCR-mediated cell spreading. We argue that ΔCT246 and ΔCT258 Cx43-expressing cells result in decreased BCR-induced pTyr because of a loss of ERM regulation by the Cx43 CT and/or Cx43-ERM binding, which affects BCR cluster formation and signaling. The same reasons may explain why these mutants do not support BCR-mediated cell spreading, given that an ERM-Cx43 interaction could be important for membrane remodeling that drives cell spreading. ΔCT307 is likely able to allow cell spreading (and pTyr) even though it lacks the ERM-Cx43 binding site because it still contains critical residues, e.g. tyrosines (and/or the serines presented here), within the region between aa 258-307 that has been shown to be important for cell spreading (Pournia, 2015). This is supported by the fact that S279A and S282A Cx43-expressing mutants do contain the ERM-binding motif but are still unable to support BCR-mediated cell spreading and pTyr, highlighting the importance of residues in this region for both of these cellular processes.
Table 4-1. Comparison of the Cx43 CT’s effect on various cellular processes

<table>
<thead>
<tr>
<th>Cell type (J558µm3 cells)</th>
<th>Tyrosine phosphorylation of proteins in response to BCR signaling</th>
<th>Potential binding to ERM proteins</th>
<th>BCR-mediated cell spreading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype Cx43</td>
<td>Control</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>ΔCT246 Cx43</td>
<td>Decreased</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>ΔCT258 Cx43</td>
<td>Decreased</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>ΔCT307 Cx43</td>
<td>Delayed at early time points; similar to wildtype Cx43 later</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>S255A Cx43</td>
<td>No change</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>S262A Cx43</td>
<td>No change</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>S279A Cx43</td>
<td>Decreased</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>S282A Cx43</td>
<td>Decreased</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>S255, 262, 279, 282A (4S)</td>
<td>No change</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
4.3 Future Directions

Direct assessment of the role of Cx43 in BCR-mediated Rap1 activation is hindered by the fact that Rap1 can be activated by physical force in J558µm3 cells. To circumvent this, the activity of upstream regulators or downstream effectors of Rap1 could be considered instead. CalDAG-GEF is a guanine exchange factor specific for Rap1 and mediates calcium/DAG-dependent Rap1 activation (Kawasaki et al., 1998). The activity of CalDAG-GEF can be easily monitored by western blotting as CalDAG-GEF is regulated by phosphorylation on S587 by PKA, which inactivates CalDAG-GEF upon its phosphorylation (Subramanian et al., 2013). Likewise, the actin-severing protein cofilin has been shown to be regulated by Rap1 (Wang et al., 2017) and so revisiting cofilin activity in the context of ΔCT246 Cx43 would be worthwhile. Both CalDAG-GEF and cofilin would infer Rap1 activity.

The cell stretching system in which cells are bound to silicone sheets via anti-MHC I antibodies can be used in the future with other cell types as an alternative method for cell adherence in lieu of ECM proteins. Other parameters of cell stretching (e.g. uniaxial stretching) or readouts after stretching can also be tested further in J558µm3 cells. Since Rap1 is activated by mechanical stress, it would be interesting to see how Rap1 reacts to tensile stress in these cells and how Cx43 may be affecting this response. Intracellular signaling assays on stretched cells would yield additional clues into how the cytoskeleton is behaving that will complement phenotypic assays.

BCR-mediated tyrosine phosphorylation of proteins was consistently different in cells expressing wildtype Cx43 versus those expressing ΔCT Cx43 and Cx43 serine-to-alanine mutants. More importantly, this result granted us a starting point in which we could reliably assay a large panel (e.g. 20+) of additional Cx43 mutants. These mutants hold immense amounts of information regarding the function of specific residues within the Cx43 CT and BCR-mediated protein tyrosine
phosphorylation can give us clues as to which mutants would benefit from further investigation. Other signaling assays for specific proteins of interest can be revisited at this point with modified methods. Finally, signaling results in identified mutants can be paired with phenotypical assessments like BCR-induced cell spreading or chemokine-driven cell migration to draw a more complete picture of the role of Cx43 in B cell cytoskeletal-dependent processes.

In parallel, phosphorylation of the ERM proteins in response to BCR signaling was shown to be impaired in the absence of the Cx43 CT. It is speculated that the CT normally serves as a platform to recruit ERM regulatory proteins and/or interact with ERM proteins themselves. The next steps would be to demonstrate whether the activity of such regulatory proteins differ upon the removal of Cx43 and whether they localize or interact with the Cx43 CT. SLK has been discussed but in lymphocytes LOK and PKC have also been reported to activate ERM proteins via phosphorylation (Belkina et al., 2009; Pietromonaco et al., 1998). Lastly, it may be interest to explore the implications of a Cx43-ERM interaction in B cells. Spreading is actin-dependent (Fleire et al., 2006) and ERM proteins are known to be important for lamellipodium formation (Baumgartner et al., 2006). Could this interaction be important during Cx43-promoted, BCR-mediated cell spreading in J558µm3 cells? Cx43 and Ezrin has been shown to interact in human trophoblasts (cells forming the outer layer of a blastocyst) (using Co-IP and Immunofluorescence) (Pidoux et al., 2014) and rat liver epithelial cells (using Immunofluorescence and Proximity Ligation Assays) (Dukic et al., 2017a) but has not been demonstrated in B cells. Co-IP and immunofluorescence localization experiments can be performed in J558µm3 B cells to first verify an interaction exists. If so, what are the effects of breaking this interaction? Ezrin, Radixin, Moesin proteins can be knocked down, but to avoid global disruption of cellular functions in general, it is possible to solely target the Ezrin-Cx43 binding site. A single amino acid at R370 of the Cx43 CT was identified to be absolutely essential for Ezrin binding (Dukic et al., 2017b) and therefore site-directed mutagenesis can be used to mutate this residue specifically. Having these mutants, cell morphology and cytoskeleton-driven cell processes such as BCR-mediated cell
spreading can be assayed to understand how Cx43 affects B cytoskeleton remodeling through ERM proteins.

### 4.4 Conclusions

This thesis aimed to understand the role of Cx43, in particular its carboxyl tail, on BCR signaling responses. It has found that in addition to activation via the BCR signaling pathway, the Rap1 GTPases can be activated by mechanical stress applied to J558μm3 plasmacytoma B cells. It has also demonstrated that the region between aa 246-307 of the Cx43 CT, as well as S279 and S282, but not S255 and S262, was important for the tyrosine phosphorylation of proteins following BCR signaling, indicating that the Cx43 CT had a global effect on the BCR signaling pathway and that specific amino acids exhibit differential properties. Cell stretching experiments suggested that the role of Cx43 in B cell cytoskeleton remodeling was through promoting intracellular signaling through the BCR. In line with this, phosphorylation of the ERM actin-plasma membrane tethering proteins were found to be attenuated at a cell basal state as well as in response to BCR signaling when the Cx43 CT was lost. This pointed to a possible role for Cx43 in regulating BCR-induced cytoskeleton rearrangements through controlling the activity of the ERM proteins. Our work may reveal Cx43 as a key protein in the B cell activation process and could contribute to future understanding of aberrant cellular cytoskeleton reorganization underlying autoimmune diseases and cancer metastasis.
References


channel protein, gap junction connexin43, occurs after exit from the ER. Cell 74, 1065-1077.

receptor signaling. Curr Opin Immunol 12, 256-266.


Neijssen, J., Pang, B., and Neefjes, J. (2007). Gap junction-mediated intercellular communication in

Nelson, W.J. (2009). Remodeling epithelial cell organization: transitions between front-rear and
apical-basal polarity. Cold Spring Harb Perspect Biol 1, a000513.

Nishimura, S., Seo, K., Nagasaki, M., Hosoya, Y., Yamashita, H., Fujita, H., Nagai, R., and Sugiura,

guanine nucleotide releasing protein 3 in coupling phospholipase C-gamma2 to Ras in B cell receptor

258.


substrate that connects B cell receptor to phosphoinositide 3-kinase activation. Immunity 13, 817-827.

Shear stress stimulation of p130(cas) tyrosine phosphorylation requires calcium-dependent e-Src

function of the inflammatory smooth muscle cell phenotype in atherosclerosis. J Vasc Res 47, 168-
180.

system: differential expression of connexin40 and 43, and perturbation of gap junction channel
functions in peripheral blood and tonsil human lymphocyte subpopulations. Immunology 99, 578-
590.


Appendices

A1. The role of the Cx43 CT in phosphorylation of BLNK, Akt, and FER proteins.

BLNK, Akt, and FER proteins were also examined for differences in activity upon loss of the Cx43 CT. These experiments were done once and stopped due to problems with protein detection. The BLNK scaffolding protein and Akt pro-survival kinase are major signaling proteins activated upon BCR signaling (Abraham et al., 2016). BLNK phosphorylation appeared at 1 min and continued until the 15 min time-point in ΔCT246 Cx43-expressing cells, but was undetectable in wildtype Cx43 J558µm3 cells (A1a). Hence, no comparison could be made. Likewise, phospho-Akt was only barely detectable in either cell types (A1b) and no conclusions were drawn. FER is a non-receptor tyrosine kinase that has been reported to be phosphorylated (potentially by Lyn kinase (Udell et al., 2006)) in response to BCR signaling (Harumiya et al., 2013). This protein may play roles in regulating cytoskeletal rearrangements (Craig and Greer, 2002). It was unclear whether the protein bands shown in A1c are background bands or actual phosphorylated FER proteins. If they were phospho-FER proteins, then there was no difference between wildtype Cx43 and ΔCT246 Cx43-expressing J558µm3 cells. Total FER was not examined because anti-mouse FER antibodies were not available.
A1. Cx43 CT in phosphorylation of BLNK, Akt, and FER proteins.
J558µm3 cells expressing wildtype Cx43 and ΔCT246 Cx43 were stimulated with 20 µg/ml anti-mouse IgM antibodies for the indicated times. The zero time-point is a control sample stimulated similarly with PBS alone. Lysates were examined by western blot analysis. A: Anti-phospho-BLNK and anti-BLNK antibodies were used to probe for BLNK activity. B: Anti-phospho-Akt and anti-Akt antibodies were used to probe for Akt activity. C: Anti-phospho-FER antibodies were used to probe for changes in FER phosphorylation after BCR signaling. Each protein had been tested once and the western blots are shown.