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Role of RhoA in B cell development and transformation of B cell precursors

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Table of Contents

Abbreviation iii
1 Introduction 1
1.1 The immune system 1
1.2 B cell development 2
1.3 B cell receptor (BCR) signalling 9
1.4 Small GTPases 12
1.5 B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) 21
1.6 Aim of the study 23
2 Material and methods 24
2.1 Materials 24
2.1.1 Equipments- General 24
2.1.2 Disposables 25
2.1.3 Chemicals 27
2.1.4 Reagents 28
2.1.5 Media and buffers for cell culture 29
2.1.6 Solutions and buffers 32
2.1.7 Media for bacterial culture 39
2.1.8 Bacterial strains 41
2.1.9 Retroviral expression vectors used in the study: 41
2.1.10 Primers used in the study 41
2.1.11 Cell lines used in the study 42
2.1.12 Antibodies 43
2.1.13 Kits 44
2.1.14 Softwares 45
2.2 Methods 46
2.2.1 Mice 46
2.2.2 Cell culture 47
2.2.3 Flow cytometry 49
2.2.4 Plasmids and retroviral transduction 50
2.2.5 Protein methods 50
2.2.6 Molecular biology and biochemical methods 52
2.2.7 Statistical Analysis 55
3 Results 56
3.1 GEF-H1 is a novel interaction partner of SLP-65 56

3.2 SLP-65 interacts with RHOA and regulates its activation 57
3.3 RHOA connects SLP-65 to PI3K-PTEN signalling 59
3.4 RHOA is essential for early B cell development 60
3.5 RHOA is important for proper development of mature B cells and generation of antibody responses 69
3.6 Significance of RHOA in precursor B cell acute lymphoblastic leukaemia (BCP-ALL)… 72
4 Discussion 77
4.1 Small GTPase RHOA connects SLP-65 activation to PI3K signalling and is important for early and mature B cell development 77
4.2 RhoA is required for the survival and migration of BCR-ABL1 transformed ALL cells……. 80
5 Summary 82
6 Bibliography 83
Acknowledgement 102
Appendix 103
Curriculum Vitae 104

Abbreviation

4-OHT	4-hydroxytamoxifen
Ab(s)	Antibodies
ADP	Adenosine di-phosphate
AID	Activation-induced cytidine deaminase
Akt/PKB	Serine/threonine protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanin 7
APC(s)	Antigen-presenting cell(s)
APS	Ammonium persulfate
ARHGEF	Rho Guanine nucleotide exchange factor
AU	Arbitrary unit
B220/CD45R	220 kDa full length isoform of tyrosine phosphatase CD45
BAD	Bcl-2-associated death promoter
BAFF	B cell-activating factor
BAFFR	B cell-activating factor receptor
B-ALL	B-cell acute lymphoblastic leukemia
BASH	B-cell adaptor containing Src-homology 2 domain
BAX	Bcl-2-associated X-protein
BCAP	B-cell PI3K adaptor protein
Bcl2	B-cell lymphoma protein 2
Bcl-XL	B-cell lymphoma extra large
BCL6 / Bcl6	B-cell lymphoma protein 6
BCP-ALL	B-cell precursor acute lymphoblastic leukemia
BCR	B-cell antigen receptor
BCR-ABL1	Break point cluster region-Abelson tyrosine kinase 1
BLIMP-1 / Blimp-1	B lymphocyte-induced maturation protein-1
BLNK	B-cell linker protein
bm	Bone marrow
Bp(s)	Base pair(s)
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
°C	Degree Celsius
Ca^{2+}	Calcium
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CDC42	Cell division cycle 42
C_H	Constant domain of immunoglobulin heavy chain
C_L	Constant domain of immunoglobulin light chain
CIP	Calf intestinal phosphatase
cKit	CD117 mast/stem cell growth factor receptor

CLL	Chronic lymphocytic leukemia
CLP(s)	Common lymphoid progenitor(s)
cm	Centimeter
CML	Chronic myeloid leukemia
CNS	Central nervous system
Cre	Causes recombination/cyclization recombinase
Cre-ER^{T2}	Cre recombinase fused to triple-mutated estrogen receptor
CRLF2	Cytokine receptor-like factor 2
CSK	C-terminal Src tyrosine kinase
CSR	Class switch recombination
CT04	Cell permeable Rho Inhibitor
ctrl	Control
CXCL12	C-X-C motif ligand 12
CXCR4	C-X-C chemokine receptor 4
Cy	Cyanin
D	Diversity
DAG	Diacyl glycerol
DAPI	4’,6-diamidine-2’-phenylindole dihydrochloride
Dbl	Diffuse large B-cell lymphoma like
DC(s)	Dendritic cell(s)
DG75-EB	Human Burkitt lymphoma cell line
D_h	Diversity gene segment of the immunoglobulin heavy chain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
DTT	Dithiothreitol solution
DZ	Dark zone
EBF	Early-B cell factor
EDTA	Ethylenediaminetetraacetic acid
eF	eflour
ELISA	Enzyme-linked immunosorbent assay
ELP(s)	Early lymphoid progenitor(s)
ER	Endoplasmic reticulum
ER	Estrogen receptor
ER^{T2}	Mutated form of estrogen receptor ligand binding domain
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
ETP(s)	Early T-cell progenitor(s)
E2A	E box binding protein
EV	Empty vector
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FcγRIIB	Fc receptor IIB for IgG
FDC	Follicular dendritic cells
FITC	Fluorescein isocyanate
floxed	Flanked by loxP sites

fl/fl	Floxed allele
FLT3/Flt3	Fms-like tyrosine kinase receptor 3
FO	Follicular
FoxO1	Forkhead box transcription factor O1
FSC	Forward scatter
FVD	Fixable viability dye
g	Gravitational acceleration
GAP(s)	GTPase activating proteins
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GC	Germinal center
γC	Gamma chain
GDI(s)	Guanine nucleotide dissociation factors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GEF-H1	Guanine nucleotide exchange factor-H1
GFP	Green fluorescent protein
GFP+	Green fluorescent protein positive
G-LISA	G-protein linked immunosorbent assay
gm	Gram
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GSK3ß	Glycogen synthase kinase-3ß
GTP	Guanosine triphosphate
GTPase	Enzyme hydrolyzing guanosine triphosphate
h	Hour(s)
HC	Heavy chain
HCl	Hydrochloric acid
HEK293T cell(s)	Human embryonic kidney cell(s)
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
Hsp(s)	Heatshock protein(s)
IFN	Interferon(s)
Ig	Immunoglobulin(s)
Ig-α	B-cell antigen receptor complex-associated protein α-chain/CD79a
Ig-β	B-cell antigen receptor complex-associated protein β-chain/CD79b
IgH	Immunoglobulin heavy chain gene locus
IgL	Immunoglobulin light chain gene locus
IKK	IkB kinase complex
IKZF1	Ikaros family zinc finger protein 1
IL	Interleukin
IL-7R	Interleukin-7 receptor
IP	Immunoprecipitation
IRES	Internal ribosome entry site
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif

IkBa	Inhibitor of NF-kB
J	Joining
J_H	Joining gene segment of the immunoglobulin heavy chain
JAK	Janus kinase
JNK	c-Jun NH2-terminal kinase
k	Kilo
kb	Kilo base pair
kDa	Kilo Dalton
l	Liter
L5	Lambda5
LC	Light chain
LNK	Lymphocyte adapter protein
LMPP(s)	Lymphoid primed multipotent progenitor(s)
loxP	Locus of X-over P1
LPS	Lipopolysaccharide(s)
LZ	Light zone
M	Molar
MACS	Magnet-activated cell sorting
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MCL-1	Myeloid cell leukemia sequence-1
MEK	MAPK/ERK kinase
MFI	Mean fluorescene intensity
mDia	Mammalian homolog of Diaphanous
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
mIg	Membrane bound immunoglobulin
ml	Milliliter
mM	Millimolar
mm	Millimeter
mRNA	Messenger ribonucleic acid
MSCV	Murine stem cell virus
mTOR / mTor	Mammalian target of rapamycin
MPP(s)	Multi potent progenitor(s)
MZ	Marginal zone
ns	Not significant
N-FAT	Nuclear factor of activated T cells
NF-kB	Nuclear factor k light chain enhancer of activated B cells
ng	Nanogram
nm	Nanometer
NH4Cl	Ammonium chloride
NHEJ	Non-homologous end joining
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl hapten
NK cell(s)	Natural killer cell(s)
NOD	Nonobese diabetic

NP(24) KLH	4-hydroxy-3-nitrophenylacetyl(24)-keyhole limpet hemocyanin
PAMP(s)	Pathogen associated molecular pattern(s)
Pax5	Paired box gene 5 transcription factor
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PBX1	Pre-B cell leukemia transcription factor 1
PCR	Polymerase chain reaction
PDK1	3-phosphoinositide-dependent protein kinase 1
PDX	Patient derived xenografts
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanin 7
PerCP	Peridinin-chlorophyll-protein
PH	Pleckstrin-homology (domains)
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)- or (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase c
PKN	Protein kinase N
PK1	P21 activated kinase 1
PLA	Proximity ligation assay
PLC-g2	Phospholipase C-g2
pMI	Plasmid MSCV-IRES
pMIG	pMI-GFP
pNPP	4-nitrophenyl phosphate
PRR	Pattern recognition receptor
Pre-	Precursor
Pro-	Progenitor
PtC	Phosphatidylcholine
PTEN / Pten	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Rag	Recombination-activating gene
Ras	Rat sarcoma small GTPase
Rho	Ras homolog
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROCK	Rho associated coiled-coil containing kinase
Rpm	Revolutions per minute
RPMI (medium)	Roswell Park Memorial Institute (medium)
RSS	Recombination signal sequence(s)
RT	Reverse transcriptase
RQ	Relative quantification
RU(s)	Relative unit(s)
S phase	Synthesis phase of the cell cycle
SCID	Severe combined immune deficiency
SD	Standard deviation
SDF-1	Stromal derived factor-1

SFK	Src-family (tyrosine) kinase
SH	Src-homology
SHM	Somatic hyper mutation
SHIP-1 / Ship-1	Src-homology region 2 (SH2) domain-containing inositol phosphatase 1
SHP-1 / Shp-1	Src-homology region 2 (SH2) domain-containing phosphatase 1
SH2B3	SH2B adapter protein 3
SLC	Surrogate light chain
SLP-65	Src-homology region 2 (SH2) domain-containing leukocyte protein of 65 kDa
Src	Sarcoma
SRp20	Serine- and arginine-rich splicing factor 3 (SRSF3)
SSC	Sideward scatter
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TCL	Total cell lysate
TCR	T-cell antigen receptor
TD	T-cell dependent
TdT	Terminal deoxynucleotidyl transferase
Tfh cell(s)	Follicular T helper cell(s)
Th cell(s)	T helper cell(s)
TI	T-cell independent
TKI(s)	Tyrosine kinase inhibitor(s)
TKO	Triple knock-out
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TSLP	Thymus stroma lymphopoietin
TSLPR	Thymus stroma lymphopoietin receptor
U	Units
V	Variable
Vav	Guanine nucleotide exchange factor
V_H	Variable gene segment of the immunoglobulin heavy chain
WT	Wild-type
µg	Microgram
µl	Microliter

Results presented in this dissertation have been previously published in the following publication

- **The small GTPase RHOA links SLP-65 activation to PTEN function and is essential for the generation and survival of normal and malignant B cells**

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1 Introduction

1.1 The immune system

All multicellular organisms possess a highly sophisticated and evolutionarily developed defence mechanism called the immune system, which protects them from foreign pathogens and toxins. In vertebrates, two distinct branches of the immune system, namely innate and adaptive immunity, carry out immune responses. Both these systems work closely together and take on distinct functions. Innate immunity is the body's first line of defence against pathogens and responds in a less-specific manner (Janeway and Medzhitov, 2002). Innate immune receptors are germline-encoded and are not clonally distributed (Vivier and Malissen, 2004). They recognize evolutionarily conserved molecules on microorganisms through the help of pattern recognition receptors (PRRs) which include Toll-like receptor (TLR) family (Beutler, 2004). PRRs recognize unique microbial components known as pathogen-associated molecular patterns (PAMPs) such as Lipopolysaccharides (LPS) or bacterial CpG DNA and directly activate innate immune cells to initiate an immune response (Akira et al., 2006; Vivier and Malissen, 2004). The innate immune system includes phagocytic cells (neutrophils, macrophages, dendritic cells (DC), and monocytes), cells that release inflammatory mediators (mast cells, neutrophils, eosinophils, basophils) and natural killer (NK) cells (Janeway and Medzhitov, 2002; Raulet, 2004). The innate immune response is also carried out by skin and epithelial cells of the respiratory, gastrointestinal and genitourinary tract. Additionally, the innate response also includes soluble proteins and bioactive small molecules that are constitutively present in biological fluids (complement proteins) or released from the cells as they are activated (cytokines, chemokines, reactive free radical species) (Chaplin, 2010; Turvey and Broide, 2010). The Innate immune system plays a significant role in activating subsequent adaptive immune responses (Parkin and Cohen, 2001).

In contrast to the innate immunity, the adaptive immune system can generate highly specific immune reactions against invading pathogens. Major cells of the adaptive immune system are T lymphocytes that mature in the thymus and antibody-producing B-lymphocytes which originate from bone marrow (Boehm, 2011; Chaplin, 2010). Adaptive immune response primarily depends on antigen-specific receptors which are expressed on the surface of B and T cells each having unique specificity for different antigens. T and B lymphocyte antigenic receptors (TCR and BCR) are generated by the somatic recombination of germline-encoded variable (V), diversity (D) and joining (J) segments. Cellular adaptive immunity is mediated by T lymphocytes expressing αβTCR which recognizes the peptide antigen presented in a complex with major histocompatibility complexes I (MHC-I) or II molecules (Marshall et al., 2018). Furthermore, there are γδ and Natural killer T cells which recognize antigens presented by non-classical MHC molecules of the CD1 family (Bonilla and Oettgen, 2010). The antigen presentation process stimulates T cells to differentiate into cytotoxic T cells ($CD8^+$) or helper T cells ($CD4^+$) (Chaplin, 2010). Cytotoxic T cells are activated by the interaction of TCR with MHC class I molecules, leading to the production of effector cells that induce apoptosis of the target antigen (Bonilla and Oettgen 2010). CD4+ helper T (Th) cells are activated once the TCR recognizes antigen bound to MHC class II proteins and thereby mediate both humoral and

cellular immune responses. Once stimulated, Th cells start to produce cytokines and differentiate into Th1, Th2 and Th17. Th1 secret interferon-gamma (IFN-γ) which activates macrophages and cytokine interleukin-2 (IL-2) which contributes to the differentiation of B cells to produce antibodies (Marshall et al., 2018). Cytokines (IL-4, IL-5 and IL-13) produced by Th2 cells recruit mast cells and eosinophils and enhance Immunoglobulin E (IgE) production (Chaplin, 2010). Furthermore, Th17 produce IL-17 and drive granulocyte-mediated immune response (Bonilla and Oettgen, 2010). In addition, the $CD4^+$ regulatory T cells (Treg) function to control immune response against self-antigens and thereby prevent autoimmunity (Marshall et al., 2018). Adaptive humoral immunity is mediated by antibodies produced by plasma cells that develop from B cells which are activated either upon signals received from T cells or by the binding of polymeric proteins with repeating structures (Bonilla and Oettgen, 2010; Chaplin, 2010). Generally, activation of B cells can lead to two different outcomes depending on the type of antigen, the place of infection as well as the affinity of the BCR towards the antigen. It is thought that B cells with BCRs of high affinity for the antigen directly differentiate into short-lived plasma cells secreting antibodies of the IgM isotype. B-lymphocytes recognizing their antigen with intermediate affinity undergo a process termed germinal centre (GC) reaction (Nutt et al., 2015). During GC reaction B cells increase their affinity for the antigen and change the isotype of their BCR through activation-induced cytidine deaminase (AID)-dependent processes known as somatic hypermutation and class switch recombination, respectively (Li et al., 2004). After GC reaction, B cells develop either into long-lived plasma cells secreting high-affinity antibodies of the IgG, IgA or IgE isotype or develop into memory B cells that persist even years after primary infection and are in place to enable a fast and effective immune response upon the second encounter with the same pathogen (Nutt et al., 2015).

1.2 B cell development

In mammals, B cells are generated from hematopoietic stem cells in the foetal liver before birth and in the bone marrow after birth. B lymphopoiesis is characterized by the expression of various cell surface markers, lineage-specific transcription factors along with sequential rearrangement of immunoglobulin (*Ig*) gene segments ultimately leading to the expression of antigen-specific B cell receptor (BCR) (Hardy and Hayakawa, 2001). BCR expressing mature B cells migrate to peripheral lymphoid organs such as the spleen, and lymph node where they differentiate into antibody-producing plasma cells or memory B cells upon antigen encounter (Alt et al., 1987).

1.2.1 Early B cell development

B cell development takes place in the bone marrow where cell surface receptors and transcription factors guide pluripotent hematopoietic stem cells to differentiate to progenitor B cells. HSCs expressing tyrosine kinase receptor (c-kit) first give rise to uncommitted multipotent precursors (MPPs), which can differentiate either into myeloid or lymphoid lineage (Busslinger, 2004). Fms-like tyrosine kinase receptor 3 (Flt3) expressing MPPs differentiate into early lymphoid progenitor (ELP) which give rise to early T-cell progenitor (ETP) in the thymus and common lymphoid progenitor (CLP) which eventually differentiate to B, T and NK cells in the bone marrow. Mice lacking Flt3 and c-kit have severely reduced hematopoietic

cells together with reduced frequency of lymphoid progenitors (Mackarehtschian et al., 1995). CLPs express both c-kit and Flt3 along with an important B cell marker, interleukin 7 receptor (IL-7R). IL-7R is a heterodimer composed of IL-7Rα protein and common cytokine receptor γ chain, which is activated by the binding of cytokine, interleukin-7 (IL-7) produced by stromal cells. Mice lacking IL-7R or IL-7 show almost complete block of B cell development where CLPs are severely compromised in their differentiation to early pro B cells (Carvalho et al., 2001; Miller et al., 2002; Peschon et al.; Yon Freeden-Jeffry et al.). Furthermore, Flt3 and IL-7R knock out mice lack B-lymphocytes in the bone marrow (J Voβhenrich et al., 2003). This shows the significance of c-kit, Flt3, and IL-7R signalling in the generation of precursor B lymphocytes in the bone marrow of adult mice. In addition to chemokines, B-cell lineage specification and commitment are governed by a network of transcription factors including PU.1, IKAROS, E box binding protein (E2A), and Early B cell Factor 1(EBF1). PU.1 is a member of the ets-family of transcription factors which is required for the generation of both lymphoid and myeloid lineage (Medina et al., 2004; Scott et al., 1997). It has been proposed that a low concentration of PU.1 in multipotent progenitors (MPPs) favours B cell development (DeKoter and Singh, 2000). PU.1 deficiency results in the absence of B lineage progenitors expressing early B lineage genes (DeKoter et al., 2002; Scott et al., 1997). Another important transcription factor that regulates early B cell development is Ikaros, a zinc finger protein responsible for the generation of all lymphoid lineages. Similar to PU.1, Ikaros regulates the lineage potential and cell fate of lymphoid primed multipotent progenitors (LMPPs) (Georgopoulos et al., 1994; Ng et al., 2009; Yoshida et al., 2006). E box binding protein, E2A regulates the expression of key B-cell specific genes which are essential for driving the early B cell development (Bain et al., 1994; Zhuang et al., 1994). Moreover, in early B cells, E2A function cooperatively with Early B cell Factor1 (EBF1), which is a B cell-specific protein that regulates B cell differentiation and commitment by regulating the expression of multiple B cell-specific genes (Seet et al., 2004).

1.2.1.1 V(D)J recombination

B cell antigen receptors are membrane-bound immunoglobulins (Ig) molecules, composed of 2 identical heavy (H), 2 identical light chains (L), and one Igα/Igβ signal transduction heterodimer (Bankovich et al., 2007; Papavasiliou et al., 1995). All heavy chains are expressed from the *IgH* locus whereas light chains are expressed from one of the two loci, *Igk* or *Igλ*. Both heavy and light chain consists of an N-terminal antigen-binding variable (V) domain and a C-terminal constant (C) domain (Schroeder et al., 2010; Taussig, 1988). The constant domains of heavy and light chains (C_H and C_L respectively) determine the effector functions and define chain type or classes (Tonegawa, 1983). Antigen-specific variable domains are generated by a process known as V(D)J recombination in which variable regions of the heavy chain is assembled from germline variable (V), diversity (D) and joining (J) segments whereas light chain variable regions are generated from V and J segments respectively (Bassing et al., 2002). V(D)J recombination is first initiated at the *IgH* locus and later on in the light chain (*IgL*) locus (Figure 1) (Bassing et al., 2002). In mice, the *IgH* locus consists of about 100 V_H segments with the functional coding sequence, 10-15 D_H segments, and 4 J_H segments (Brodeur and Riblet, 1984; Retter et al., 2007). Similarly, the kappa light chain locus consists of 100 functional V segments and 4 functional J segments (Aoki-Ota et al., 2012). Lymphocyte-specific recombination

activating gene enzymes RAG1 and RAG2 recognize recombination signal sequences (RSS) that flank the *Ig* gene segments and induce double-stranded breaks which are later joined by non-homologous DNA end joining machinery (Bassing et al., 2002; Bogue and Roth, 1996; Ji et al., 2010; Oettinger et al., 1990). Thus, VDJ recombination of *Ig* gene segments generate an enormous diversity in BCR specificities against antigens. In addition to this, Terminal deoxynucleotidyl transferase (TdT) a lymphocyte specific enzyme adds non-germline-encoded nucleotides (N region nucleotides) to *Ig* gene segment junctions and thereby increasing the variability of the third complementarity determining region 3 (CDR3), which is one of the hypervariable regions of B cell receptor that specifically recognizes the antigen (Komori et al., 1993; Landau et al., 1987).

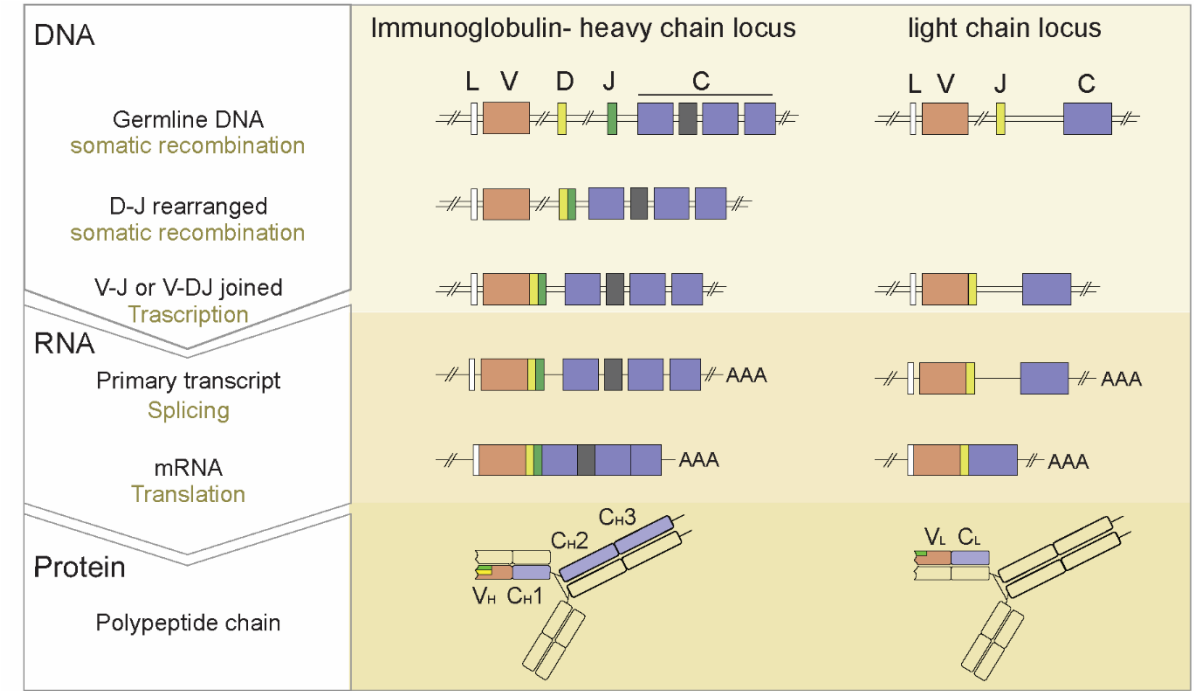

Figure 1: Schematic representation of V(D)J recombination in immunoglobulin heavy and light chain loci: The germline immunoglobulin heavy chain (*IgH*) is composed of many variable (V), diversity (D) and joining (J) gene segments upstream of the constant (C) regions. During V(D)J recombination, one D gene segment is rearranged to join one J gene segment (D-J rearrangement). Further recombination rearranges one V gene segment to join the previously rearranged DJ segment (V-DJ rearrangement), creating a functional V_H exon that encodes a functional variable domain of immunoglobulin heavy chain. The immunoglobulin light chain loci (*IgL*) *Igλ and Igκ* are composed of many variable (V), and joining (J) gene segments. V to J recombination events within these loci result in a functional light chain variable domain.
Abbreviations: AAA, polyadenylation; C, constant region; C_H, constant region of heavy chain; C_L, constant region of light chain DNA, deoxyribonucleic acid; L, leader; RNA, ribonucleic acid; V_H, variable gene segments of immunoglobulin heavy chain; V_H, variable gene segments of immunoglobulin light chain.
Figure is generated based on the information from (kenneth Murphy and Casey Weaver, 2016).

1.2.1.2 Pre-pro B cells

The earliest committed B cell precursors are pre-pro B cells (Allman et al., 1999). These cells have their *Ig* gene loci in germline configuration and are characterized by low expression of recombination activating genes *Rag1* and *Rag2* (Oettinger et al., 1990; Schatz et al., 1989).

1.2.1.3 Pro-B cells

Pro-B cells differentiate from pre-pro B cells by the expression of various surface markers and along with recombination status of Immunoglobulin heavy chain (Hardy et al., 1991). RAG-mediated immunoglobulin rearrangement is initiated on *IgH* locus at the early pro B cell stage, where D_H to J_H segment recombination occurs first followed by V_H-DJ_H rearrangement at the later pro-B cell stage (Chowdhury and Sen, 2004; Jung and Alt, 2004). D_H-J_H rearrangement takes place at both alleles in pro-B cells suggesting both IgH alleles are available for V_H-DJ_H recombination (Ehlich et al., 1994). Pro-B cells also express a precursor form of B cell receptor composed of signalling subunits Igα, Igβ along with calnexin (Nagata et al., 1997). Moreover, IL-7R/IL-7 signalling plays a crucial role in the pro-B cell stage. IL-7R signalling is not only required for the proliferation, survival, and differentiation of pro-B cells but also for the expression of B cell-specific transcription factor Pax5, which enforces the B lineage commitment of precursors (Billips et al., 1995; Corcoran et al., 1998; Miller et al., 2002).

1.2.1.4 Pre-B cells

Successful in frame V(D)J rearrangement of *IgH* chain locus at Pro-B cells leads to the assembly of pre-BCR and marks the transition to pre-B cells (Pillai and Baltimore, 1987; Tsubata and Reth, 1990). The pre-BCR complex comprises two identical μ heavy chains, two germ line encoded surrogate light chains each composed of VpreB and λ5 and signalling subunits Igα and Igβ (Karasuyama and Melchers, 1990; Lassoued et al., 1993). The importance of the pre-BCR in B cell development is demonstrated by studies of mice deficient in the expression of pre-BCR components (Ehlich et al., 1994; Kitamura et al., 1992; Lutz et al., 1998; Mårtensson et al., 1999). Despite its transient expression, membrane-bound pre-BCR serves as an important checkpoint in B cell development. Autonomous pre-BCR signalling activates many important signalling molecules which are required to induce both proliferation and differentiation of pre-B cells (Herzog et al., 2009; Ohnishi and Melchers, 2003). Signals through pre-BCR induce several rounds of proliferation thereby increasing the pool size of the large pre-B cell expressing productively recombined *IgH* gene (Hess et al., 2001; Rolink et al., 1999). Importantly, pre-BCR signals downregulate the expression of recombination activating genes *Rag1*, and *Rag2* and ensure allelic exclusion at the heavy chain locus. (Grawunder et al., 1995; Melchers, 2005). Allelic exclusion ensures that B cells are generated with a single antigen specificity, by allowing DNA recombination of only one allele at heavy and light chain locus. One of the key components that are activated downstream of pre-BCR is an adaptor protein called SLP-65 (Src homology containing leukocyte protein of 65 kDa) (Fu et al., 1998; Hashimoto et al., 1999; Wang and Clark, 2003; Wienands et al., 1998). SLP-65 downregulates the λ5 expression thereby silencing SLC gene transcription, a mechanism by which pre-BCR limits its own expression and signalling (Parker et al., 2005). Moreover, SLP-65 reactivates the *Rag* genes and induces the recombination at the IgL locus which eventually leads to the differentiation of small resting pre-B cells into immature B cells (Herzog et al., 2008).

1.2.1.5 Immature B cells

Immature B cells that have undergone productive IgL rearrangement replace the SLC with a conventional light chain (LC) and express mature B cell antigen receptor (BCR) of IgM isotype on the surface (Figure 2) (Herzog et al., 2009; Wang and Clark, 2003). Due to the random

assembly of *IgH* and *IgL* genes, BCRs expressed by immature B cells can be non-functional or autoreactive (Übelhart and Jumaa, 2015). At this stage, immature B cells are subjected to various selection processes to generate self-tolerant B cells. Immature B cells expressing autoreactive BCR, undergo secondary light chain rearrangement also known as receptor editing to generate self-tolerant BCR. B cells with exhausted receptor editing undergo either HC replacement or are removed from the repertoire by clonal deletion. Furthermore, self-reactive immature B cells that escape the above-mentioned tolerance mechanisms, are rendered anergic, a state of unresponsiveness to antigens achieved by continuous engagement of their BCR to low-avidity self-antigens (Eschbach et al., 2011; Gay et al., 1993; Goodnow et al., 1995; Nemazee and Bürki, 1989). Immature B cells that have completed central tolerance checkpoints leave the bone marrow and migrate to the spleen where they differentiate into micro anatomically and phenotypically distinct subsets of mature naïve B cells (Allman et al., 2001). Signalling through BCR plays a critical role in the bone marrow to spleen transition of immature B cells (Torres et al., 1996; Turner et al., 1997).

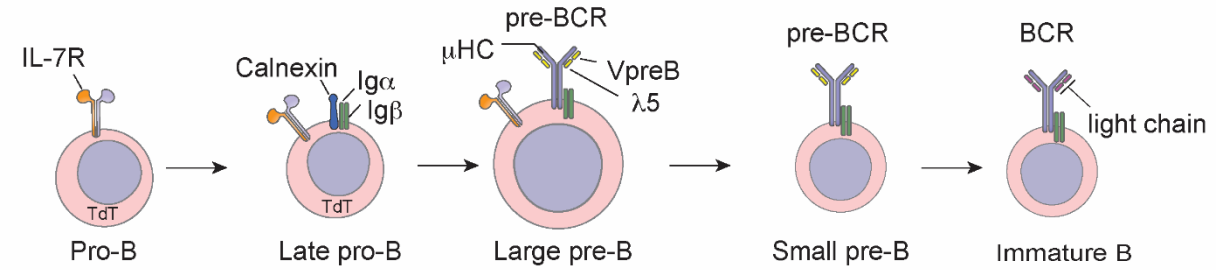

Figure 2: Schematic representation of B cell development stages. At the common lymphocyte progenitor or pro-B cell stage rearrangement of immunoglobulin heavy chain (*IgH*) is initiated and if successful, gives rise to the IgH chain that is expressed on the pre-B cell receptor (pre-BCR) at the large pre-B cell stage. Pre-BCR signalling induces clonal proliferation, allelic exclusion, down-regulation of pre-BCR components and rearrangement of the immunoglobulin light chain (*IgL*) gene. *IgL* gene rearrangements in small pre-B cells result in the expression of a BCR on the surface of immature B cells. Immature B cells undergo a selection process and eventually enter the pool of long-lived mature B cells. **Abbreviations**: BCR, B-cell receptor; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; Igα, B-cell antigen receptor complex associated protein α-chain; Igβ, B-cell antigen receptor complex associated protein β-chain; μHC, μ heavy chain; λ5, CD179b; IL-7R, interlukin-7 receptor; VpreB, CD179a; TdT, Terminal deoxynucleotidyl transferase.
Figure is generated based on the information from (Benschop and Cambier, 1999; Herzog et al., 2009).

1.2.2 Peripheral B cell development

Only 5-10 % of immature B cells (out of $2*10^7$/per day) that are produced in the bone marrow reach the spleen and enter the pool of long-lived mature B cells (Rolink et al., 1999). These new emigrant immature B cells also called transitional B cells and pass through three distinct non-proliferative stages namely transitional (T)1 ($AA4^+CD23^-sIgM^{high}$), T2 ($AA4^+CD23^+sIgM^{high}$) and T3 ($AA4^+CD23^+sIgM^{low}$) each distinguished by the relative expression of cell surface markers such as surface (s) IgM, CD23 and type I transmembrane protein AA4 (Loder et al., 1999; Allman et al., 2001). Moreover, as transitional B cells differentiate from the T1 to T2 stage they start to express BCRs of IgD isotype together with IgM and upregulate the expression of TNF alpha receptor family B cell-activating factor

receptor (BAFF-R) whose expression is crucial for survival and maturation of B cells (Loder et al., 1999; Rolink et al., 2004; Schneider et al., 2001). In contrast to mature B cells in the periphery, transitional B cells undergo apoptosis rather than proliferation upon BCR crosslinking (Allman et al., 2001). BAFF activated transitional B cells no longer enter apoptosis instead they proliferate in response to BCR activation (Rolink et al., 2004). Once completing their maturational transition, the majority of mature peripheral B cells enter the recirculating pool as naïve B cells. Naïve peripheral B cells can be divided into three distinct lineages namely B-1, Marginal zone (MZ) and Follicular (FO) B cells (Pillai et al., 2004). Cell fate decision of peripheral B cells largely depends on the strength of the signal received from the BCR. Weak or basal antigen receptor-derived signals favour follicular B cells, whereas strong signals lead to the development of marginal zone B-cell generation and even stronger BCR-derived signals generate B1-B cells (Figure 3) (Cariappa et al., 2001; Casola et al., 2004; Wen et al., 2005).

Mature naïve follicular B cells ($IgM^{low}IgD^{+}$) have a half-life of around 2-3 months and comprise the vast majority of B lymphocytes in the spleen, lymph nodes as well as in the recirculating pool of B cells in the bone marrow and blood. FO B cells enter lymphoid follicles of the spleen, lymph node and ectopic lymphoid aggregates and require productive interactions with T cells for their differentiation (Loder et al., 1999; Shlomchik and Weisel, 2012) FO B cells that engage in T cell-B cell interaction undergo proliferation and differentiation to either short-lived extrafollicular plasma blasts or long-lived Germinal centre (GC) B cells depending on their antigen affinity. Naïve FO B cells that have a low affinity for antigen start to express the transcriptional repressor B-cell lymphoma 6 (Bcl-6) and migrate to follicles and eventually form a nascent germinal centre (Dal Porto et al., 2004; Kerfoot et al., 2011; Kitano et al., 2011). GCs are specialized microenvironments where mature B cells undergo several rounds of clonal expansion and secondary diversification of their *Ig* genes thereby generating high-affinity B cells that can differentiate into antibody-producing plasma cells and memory B cells. Activated B cells, follicular dendritic cells (FDC) and follicular helper T cells (Tfh) are the three major cell types found in GCs (Shlomchik and Weisel, 2012). GCs are divided into two distinct compartments: the dark zone (DZ), where antigen-activated B cells proliferate extensively and undergo activation-induced cytidine deaminase (AID) dependent somatic hypermutation (SHM) of Ig V regions; and the light zone (LZ) where B cells expressing high-affinity antibodies undergo class switch recombination (CSR), a process by which B cells switch their Ig class from IgM or IgD to IgG, IgE or IgA and thereby elicit different effector functions (Li et al., 2004; Nutt et al., 2015; De Silva and Klein, 2015). After GC reaction, B cells differentiate into long-lived plasma or memory B cells and support humoral immune responses (Good-Jacobson and Shlomchik, 2010).

Marginal zone (MZ) B cells constitute 5-10% of total splenic B cells. They are long-lived, non-recirculating peripheral B cell subsets that resides in the marginal sinus of the spleen and respond rapidly to blood-borne pathogens (Oliver et al., 1997; Pillai and Cariappa, 2009). MZ B cells are phenotypically IgM^{high} IgD^{low} $CD21^{high}$, $CD23^{low}$, $CD1d^{high}$ and play a significant role in the immune response against T cell-independent (TI) antigens (Martin and Kearney, 2000; Oliver et al., 1997; Pillai and Cariappa, 2009). Bacteria and other particulate nature pathogens entering through blood are scavenged by specialized macrophages that reside in the marginal zone ultimately leading to MZ B cell activation (Martin and Kearney, 2000, 2002;

Viau and Zouali, 2005). Immediately after activation, MZ B cells generate effector B cells that can either present antigens (Ags) to T cells or differentiate into plasma cells that secrete antibodies of IgM isotype (Oliver et al., 1997; Pillai et al., 2004).

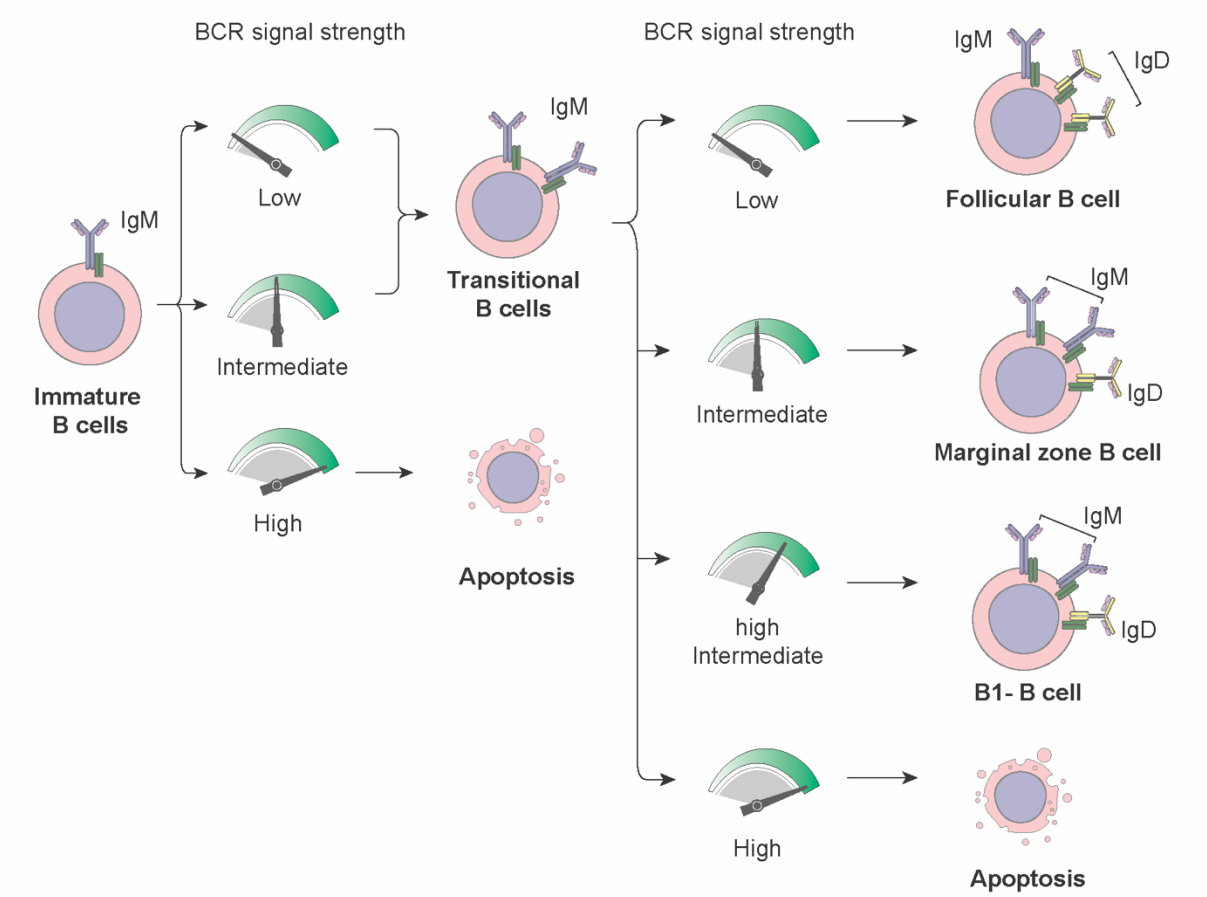

Figure 3: Schematic representation of mature B cell development in periphery: Newly formed transitional B cells are recruited to B-1, follicular or marginal zone B- cells depending on the strength of the signals emanating from B-cell antigen receptor in response to the antigen.

Abbreviations: BCR, B-cell antigen receptor; Ig, immunoglobulin.

Figure is generated based on the information from (Casola, 2007).

Besides FO and MZ B cells, the peripheral mature B cell compartments also includes a subset of long-lived, self-renewing B1 B cells. They are the predominant B cell population in peritoneal and pleural cavities (30-60%) and are also observed at a lower frequency in the spleen and other lymphoid organs (0.2-1%) (Montecino-Rodriguez and Dorshkind, 2012). Based on their CD5 expression, B1 B cells are divided into two subsets: $CD5^+$ B1a and $CD5^-$ B1b. B1a cells develop from foetal liver progenitors and participate in innate immunity whereas B1b cells are mainly developed from bone marrow-derived stem cells and are self-reactive (Hardy, 2006; Herzenberg and Tung, 2006; Pillai et al., 2004). B1 B cells exhibit less diverse V_H repertoires and have fewer N region additions at V_H-D and D-J_H Ig gene junctions due to the lack of TdT enzyme (Alugupalli and Gerstein, 2005; Martin and Kearney, 2001) Natural antibodies produced by B1 B cells are mainly of IgM isotype and characteristically have low affinity and are polyreactive. These antibodies recognize common bacterial carbohydrate antigens such as

phosphoryl choline and self-antigens including phosphatidyl choline (Ptc), DNA and Igs (Hu et al., 2002; Larry Arnold et al.; Stall et al., 1996).

1.3 B cell receptor (BCR) signalling

B cell receptor (BCR) signalling is essential for the B cell survival, development and generation of the proper immune response (Figure 4). BCR is a multiprotein complex consisting of membrane-bound immunoglobulin (mIg) which is non-covalently associated with the signalling subunit Igα (CD79a)-Igβ (CD79b) disulfide-linked heterodimer (Herzog et al., 2009). Engagement of the BCR by antigen or autonomous activation of pre-BCR results in pre-BCR/BCR aggregation which rapidly activates Src family kinases (SFKs) such as Lyn, Fyn, Blk. SFKs phosphorylate tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of Igα and Igβ (Bolen, 1995; Reth and Wienands, 1997). Phosphorylated ITAMs act as docking site and recruit the SRC homology-2 (SH2) domain-containing spleen tyrosine kinase (Syk) leading to its autophosphorylation and activation. Syk-mediated ITAM phosphorylation of neighbouring BCR complexes generates a positive feedback loop that enhances the BCR signal (Dal Porto et al., 2004; Rowley et al., 1995). Thus, Syk acts as the central molecule that couples BCR signalling to distal signal transduction elements.

Syk and Src kinases mediated phosphorylation of BCR co-receptor molecule CD19 and B-cell PI3K adaptor protein (BCAP) leads to the activation and subsequent recruitment of Phosphoinositide 3-Kinase (PI3K) to the plasma membrane (Aiba et al., 2008; Deane and Fruman, 2004; Okkenhaug et al., 2002). PI3Ks are lipid kinases which are the crucial component of BCR-induced signalosome promoting proliferation, differentiation and survival of B cells (Abdelrasoul et al., 2018; Okkenhaug et al., 2002). PI3K, upon activation, catalyses the synthesis of lipid second messenger phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) from phosphatidylinositol-(4,5)-bisphosphate (PIP2) and recruits pleckstrin homology (PH) domain-containing signalling molecules such as protein kinase B (PKB/AKT), 3-phosphoinositide-dependent protein kinase 1 (PDK1) or the Tec kinase BTK (Bruton's agammaglobulinemia tyrosine kinase) to the plasma membrane (Anderson et al., 1998; Deane and Fruman, 2004; Okkenhaug et al., 2002). PIP3-mediated BTK activation leads to phosphorylation of PLCγ2 and this connects PI3K activation to calcium signalling (Donahue and Fruman, 2004; Fruman et al., 2000).

The serine/threonine kinase AKT is the dominant mediator of PI3-K-dependent cellular proliferation and requires PDK1 and rapamycin-insensitive mTOR complex 2 (mTORC2) mediated phosphorylation for its full activation (Alessi et al., 1997; Jacinto et al., 2006; Manning and Cantley, 2007; Stokoe et al., 1997). AKT promotes the survival of B cells by regulating the activity and expression of pro and anti-apoptotic factors. Upon phosphorylation, by AKT pro-apoptotic protein BAD dissociates from anti-apoptotic Bcl2 family member, Bcl-XL (Datta et al., 2000; Del Peso et al., 1997). Furthermore, AKT signalling promotes B cell proliferation by inactivating glycogen synthase kinase 3β (GSK3β), a negative regulator of c-Myc and Cyclin D, which are important for cell cycle progression (Cross et al., 1995). In addition, AKT negatively regulates cell cycle inhibitor $p27^{kip1}$ (Fujita et al., 2002; Medema et al., 2000). One of the main targets of AKT is forkhead box class O (FoxO) transcription factors

(Greer and Brunet, 2005; Yusuf et al., 2004). Phosphorylation of FoxO proteins (including FoxO1, FoxO3a, and Foxo4) by AKT leads to their nuclear export, subsequent degradation and inactivation of their transcriptional activity (Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999). In early B cells PI3K/AKT pathway control Ig heavy and light chain recombination by regulating the expression of FoxO1 proteins, which are the key regulators of Rag-1 and Rag-2 transcription (Alkhatib et al., 2012; Amin and Schlissel, 2008; Dengler et al., 2008). PI3K signalling is not only essential for the early B cell development but also the survival and differentiation of mature B cells (Ramadani et al., 2013; Srinivasan et al., 2010; Werner et al., 2010).

Since PI3-Kinase activates many crucial downstream signalling pathways, tight regulation of PI3K-AKT signalling is mandatory to avoid uncontrolled proliferation and malignant transformation of B cells. Phosphatase and Tensin homolog (PTEN), a lipid phosphatase antagonizes PI3K signalling by dephosphorylating PIP3 (removes 3'-phosphate) to PIP2 (Leslie and Downes, 2002; Setz et al., 2019). Additionally, SH2-domain-containing inositol 5-phosphatase (SHIP), removes the 5'-phosphate to generate PI(3,4)P2 and counteracts PI3K activity (Leslie and Downes, 2002; Okkenhaug et al., 2002). Moreover, inhibition of AKT by phosphatase PHLPP (PH domain leucine-rich repeat protein phosphatase) also terminates PI3K-AKT signalling (Gao et al., 2005). Another key regulator of PI3K signalling is an adapter protein, SLP-65 (Fu et al., 1998; Hashimoto et al., 1999; Wang and Clark, 2003). SLP-65 plays a crucial role in B cell development specifically at pre-B cell differentiation shown by the increased number of pre-B cells with elevated pre-BCR expression in SLP-65 deficient mice. These mice also show partial block in B cell development with reduced mature B cells in the periphery (Flemming et al., 2003; Jumaa et al., 1999; Pappu et al., 1999). SLP-65-mediated downregulation of AKT activity leads to stabilization of FoxO proteins that induces Rag expression (Herzog et al., 2008, 2009). Thus, SLP-65 drives B cell differentiation by inhibiting PI3K signalling.

Syk-mediated phosphorylation of SLP-65 at several tyrosine residues leads to the recruitment of SH2 domain-containing signalling molecules such as Bruton's tyrosine kinase (BTK), phospholipase C gamma 2(PLCγ2), guanine nucleotide exchange factor-Vav, and growth-receptor-bound-protein 2 (Grb2) (Fu et al., 1998; Wienands et al., 1998). Thus, phosphorylated SLP-65 acts as a scaffold for effector molecule assembly. SLP-65 recruits PLCγ2 from the cytosol to the plasma membrane which leads to Syk and BTK mediated dual phosphorylation of PLCγ2(Chiu et al., 2002; Hashimoto et al., 1999; Ishiai et al., 1999). Activated PLCγ2 cleaves membrane-associated PIP2 into second messengers Inositol 1,4,5-triphosphate (IP3) and membrane-bound diacylglycerol (DAG) (Engelke et al., 2007; Kurosaki et al., 2000). IP3 binds to its receptor and leads to the mobilization of Ca^{2+} from intracellular stores such as the Endoplasmic reticulum (ER) or extracellular sites into the cytoplasm. Elevated intracellular calcium levels lead to the activation of transcriptional factors: nuclear factor of activated T cells (N-FAT) and the calcium-calmodulin pathway (Dal Porto et al., 2004; Dolmetsch et al., 1997). DAG activates Protein kinase c-β (PKC-β) and Ras guanine nucleotide release protein 3 which couples BCR activation to canonical nuclear factor-κB (NF-κB) and MAPK pathway which include extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK (Coughlin et al., 2005; Saijo et al., 2002; Su et al., 2002).

Proper regulation of the BCR signalling pathway is necessary to prevent the hyperactivity of B cells which can lead to autoimmunity. B-cell activation and signalling are tightly regulated by the activity of various non-B cell receptors (co-stimulatory and inhibitory). BCR signal initiation is indirectly regulated by two non-receptor associated molecules namely CD45, a transmembrane tyrosine phosphatase activating Src kinases such as Lyn by dephosphorylating C-terminal inhibitory tyrosine residues and C-terminal src tyrosine kinase (Csk) which acts as CD45 antagonist. Both these proteins act together for proper Lyn activation and BCR signal initiation (Benatar et al., 1996; Justement et al., 1991). CD19, a BCR-associated transmembrane protein positively regulates BCR signalling by enhancing the recruitment and activation of Lyn, PI3K, Btk and Vav (Buhl et al., 1997; Fujimoto et al., 2000). CD22, a sialic acid-binding immunoglobulin-like lectin (SIGLEC) superfamily member is a key negative regulator of BCR signalling (Nitschke et al., 2001). Lyn-mediated phosphorylation of ITIMs (immunoreceptor tyrosine-based inhibitory motif) of CD22 and FCγRIIb leads to the recruitment of SH2-domain-containing tyrosine phosphatase-1 (SHP-1) and SHIP respectively which negatively regulates BCR signalling (Blasioli et al., 1999; Famiglietti et al., 1999; Nitschke et al., 2001; Tamir et al., 2000). Thus, antigen-induced B cell receptor activation leads to the coordinated assembly of signalosomes consisting of intracellular signalling molecules as well as adaptor proteins which ultimately triggers a variety of cellular processes including regulation of gene expression, and reorganization of the cytoskeleton, and BCR-mediated internalization of antigen complexes.

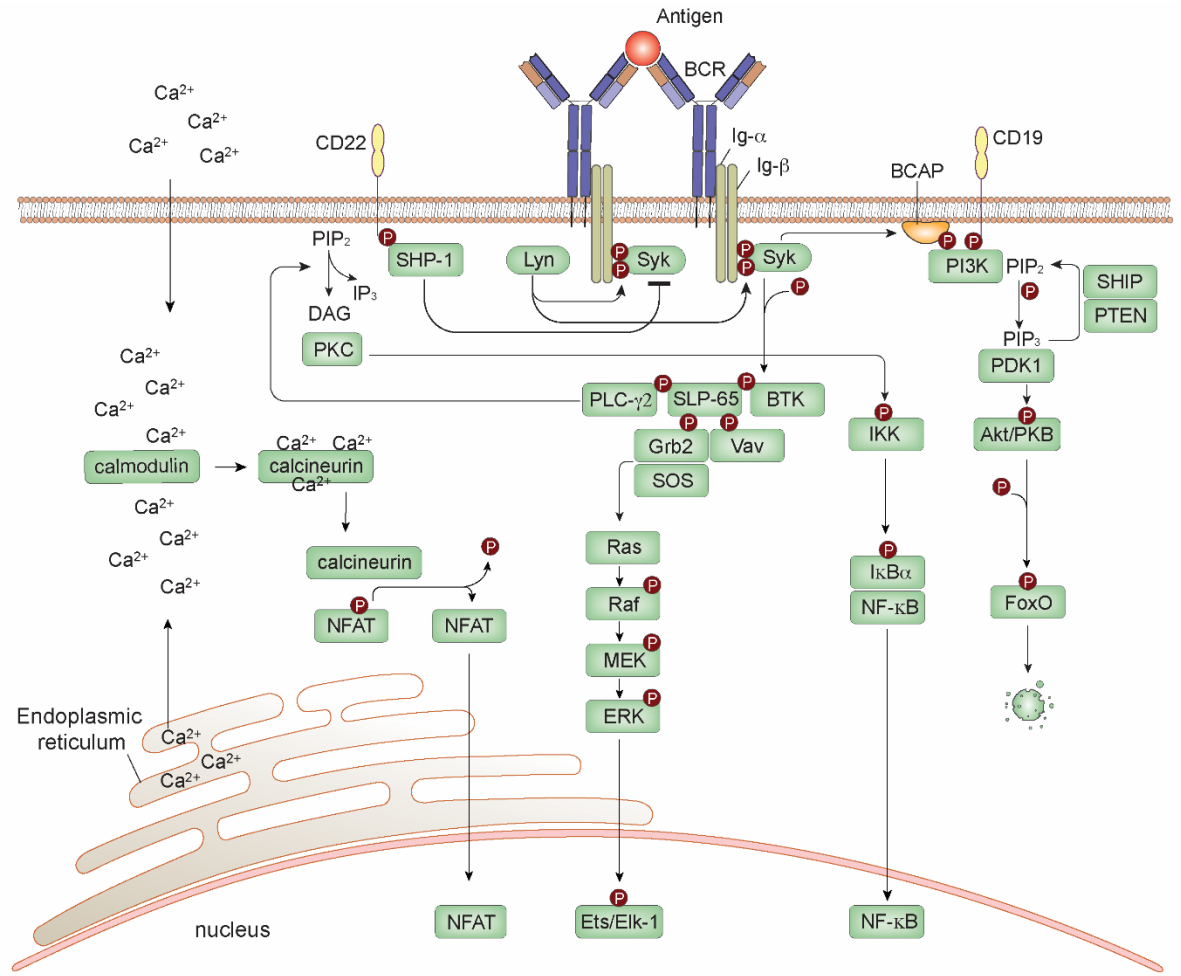

Figure 4: Schematic overview of BCR signalling: Antigen-induced engagement of the BCR induces the phosphorylation of the ITAMs in the Igα/β cytoplasmic tails and activation of the tyrosine kinase Syk, which in turn activates several downstream signalling pathways that are important for the proliferation and differentiation of B cells.
Abbreviations: BCR, B-cell antigen receptor; Syk, spleen tyrosine kinase; Lyn, src family kinase; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PIP2, phosphatidylinositol (4,5) bisphosphate; PKC, protein kinase C; BCAP, PI3K adaptor protein; PDK1, 3-phosphoinositide dependent protein kiase 1; Akt/PKB, protein kinase B; DAG, diacyl glycerol; IP3, inositol (1,4,5) trisphosphate; BTK, Bruton's tyrosine kinase; SLP-65, src homology region 2 domain containing leukocyte protein of 65 kDa; PLCγ2, phospholipase C-γ2; Ras, rat sarcoma small GTPase; Raf, rapidly accelerated fibrosarcoma serine/threonine kinase; MEK, mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK); NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κ light chain enhnacer; FoxO, forkhead box transcription factor O; Ca^{2+}, calcium; PTEN, phosphatase and tensin homolog; SHIP, SH2-domain containing inositol 5-phosphatase ; IKK, IκB kinase complex; IκBα, inhibitor alpha of NF-κB; Vav, guanine nucleotide exchange factor; Grb2, growth factor receptor bound factor 2; SOS, son of sevenless; Ets, protein C-ets-1 transcription factor; Elk-1, ETS domain containing transcription factor.
Figure is generated based on the information from (Dal Porto et al., 2004; Herzog et al., 2009; Werner et al., 2010).

1.4 Small GTPases

Small GTPases are 20-40 kDa molecular weight proteins that are commonly found in eukaryotes. They act as molecular switches in many cellular processes mainly by changing their conformation from an inactive GDP bound to an active GTP bound form (Bos et al., 2007). Depending on their structure, sequence and function, members of small GTPases are classified

into five subfamilies namely Ras, Rho, Rab, Arf, and Ran. These subfamilies are critical players in many cellular events such as cell growth, cell differentiation, cell movement and vesicle trafficking (Wennerberg et al., 2005). Members of small GTPases share 65-80% sequence similarity (Fort and Blangy, 2017; Rojas et al., 2012). This study focuses on the importance of Rho GTPases, their regulation and significance in B cell development.

1.4.1 Rho GTPases

Ras homolog (Rho) family proteins constitute a major branch of small GTPases. Mammalian Rho GTPases comprise 20 intracellular signalling proteins which play a critical role in regulating many cellular activities such as cell migration, cell cycle, vesicle trafficking, cytoskeleton rearrangement and gene expression. Members of Rho GTPases are grouped into eight subfamilies which can, in turn, be classified into two categories mainly, classical and atypical GTPases (Figure5). Classical Rho GTPases are activated by GTP binding which enables them to bind to effector molecules whereas atypical Rho GTPases are always GTP bound and are constitutively active (Heasman and Ridley, 2010; Pedersen et al., 2012).

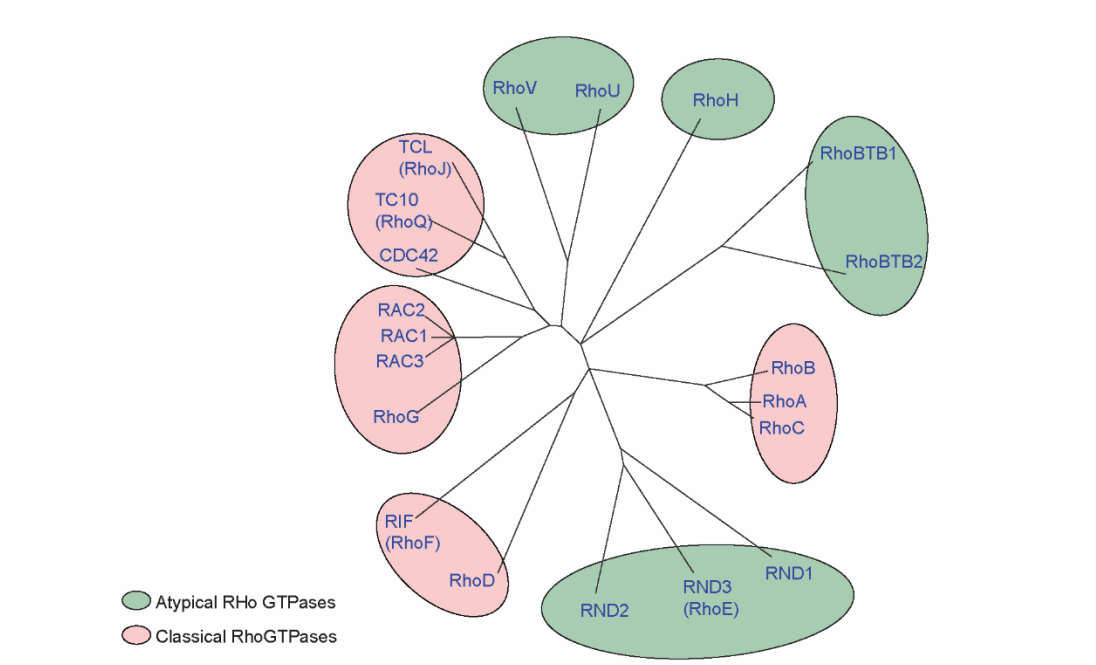

Figure 5: Rho GTPase family: Rho GTPases include 8 subfamilies which are classified into two groups. The classical Rho GTPase include the Rho, Rac, CDC42, RhoF and RhoD subfamilies whereas atypical Rho GTPases comprise RhoBTB, Rnd, RhoU and RhoV subfamilies and RhoH. Figure is generated based on the information from (Heasman and Ridley, 2008; Tybulewicz and Henderson, 2009)

1.4.1.1 Regulation of Rho GTPases

Rho GTPases act as binary switches cycling between an active GTP and an inactive GDP-bound state (Etienne-Manneville and Hall, 2002). The activity of classical Rho GTPases are determined by the ratio of the GTP/GDP bound state in the cell which are controlled by three families of proteins namely: Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and Guanine nucleotide dissociation factors (GDIs) (Figure 6) (Heasman and

Ridley, 2010). In addition to canonical Rho GTPase regulators, the Rho GTPases GTP-GDP cycle are further regulated by post translational modifications such as lipid modifications, phosphorylation, and ubiquitination (Hodge and Ridley, 2016).

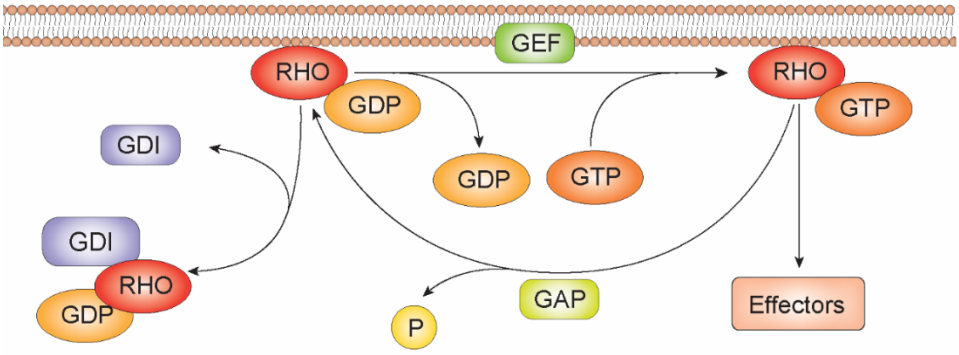

Figure 6: Regulation of Rho GTPases: Rho GTPases bind with either GDP or GTP**.** Guanine nucleotide exchange factor activates Rho GTPases by promoting the release of GDP and the binding of GTP. GTPase activating proteins (GAPs) inactivate Rho GTPases by the intrinsic GTPase activity of Rho proteins. Guanine nucleotide dissociation factors (GDIs) bind to Rho proteins and sequester them in the cytoplasm away from their regulators and effectors.
Abbreviations: GTP, guanosine triphosphate; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor, GDI, guanine nucleotide dissociation factor; GAP, GTPase activating proteins; RHO, ras homolog; P, phosphate.
Figure is generated based on the information from (Heasman and Ridley, 2008; Tybulewicz and Henderson, 2009)

1.4.1.1.1 Guanine nucleotide exchange factors (GEFs)

Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by promoting the exchange of GDP for GTP upon an internal or external stimuli (Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000; Bos et al., 2007). Human genome encodes 82 GEFs which are grouped into two families: Dedicator of cytokinesis (DOCK) related proteins and Diffuse large B cell lymphoma (Dbl) like family of proteins. In humans Dbl-like proteins are the most prominent Rho GEFs with 71 proteins identified so far. They are called Dbl-family proteins due to their similarity with Dbl (Diffuse large B cell lymphoma) protein, which is identified as an oncogene that activates cdc42 Rho GTPase (Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000; Cook et al., 2014; Rossman et al., 2005). DOCK related proteins include 11 members which are structurally and mechanistically distinct to Dbl-like family of proteins (Fort and Blangy, 2017; Rossman et al., 2005).

1.4.1.1.1.1 Guanine nucleotide exchange factor H1 (GEF-H1)

Guanine nucleotide exchange factor H1 (GEF-H1) encoded by *ARHGEF2* gene is a member of Dbl-like protein family of Rho GEFs (Krendel et al., 2002). GEF-H1 and its murine orthologue Lfc, acts as Rho GEFs exclusively for Rho subfamily but not for cdc42, Rac or Ras Rho GTPase subfamilies (Glaven et al., 1996; Krendel et al., 2002; Ren et al., 1999). Structurally GEF-H1 possesses a protein kinase C conserved region 1 (C1) domain that contains a zinc finger motif near the N-terminus followed by Dbl homology (DH) domain, Plecktrin homology (PH) domain and a unique coiled-coil domain at C-terminus (Figure 7) (Joo and Olson, 2021). The

activity of GEF-H1 is tightly regulated mainly by two processes: firstly, by regulating the access of GEF-H1 to its downstream effector RhoA and secondly by changing its activity through protein phosphorylation (Joo and Olson, 2021). GEF-H1 is localized in microtubule mainly by its N-terminal zinc finger domain (Chang et al., 2008; Krendel et al., 2002). GEF-H1 also associates with other proteins to promote its association with microtubules. Depolymerization of microtubules leads to release of GEF-HI which leads to the subsequent activation of RhoA (Birkenfeld et al., 2007; Ren et al., 1998). GEF-H1 phosphorylation at its several sites also determines its activity and localization. The phosphorylation of Threonine 678 (T678) by extracellular signal-related kinase (Erk1/2) leads to the activation of GEF-H1 whereas phosphorylation of Serine at its 959th position (S959) inhibits its activity (Fujishiro et al., 2008; Kakiashvili et al., 2009; Thun et al., 2013). Serine 885 (S885) phosphorylation by p21-actvated kinase 1 (pk1) leads to the localization of GEF-H1 to microtubules and subsequent inactivation (Zenke et al., 2004).

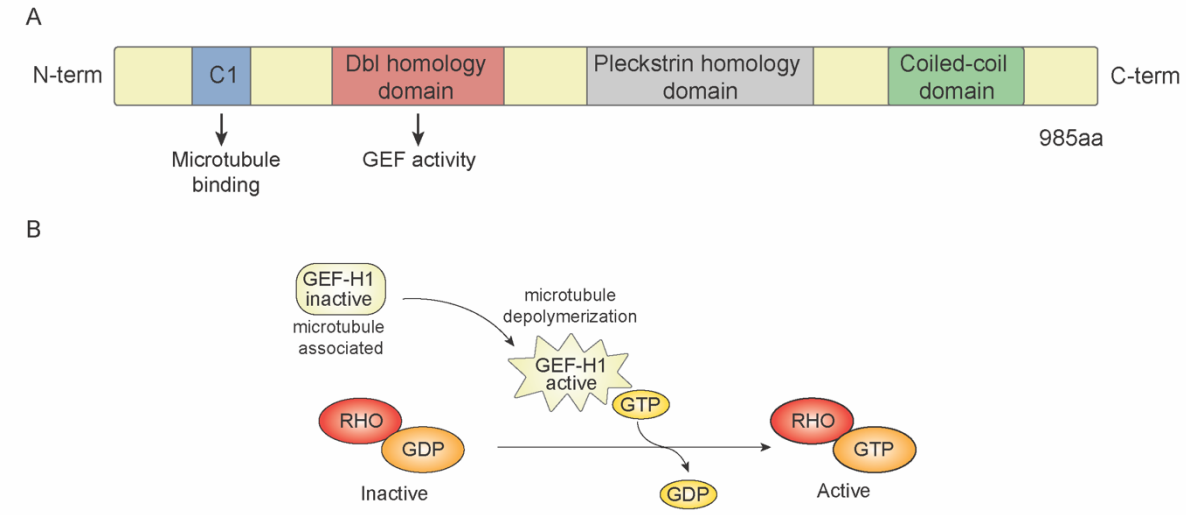

Figure 7: Structure of GEF-H1: GEF-H1 has a C1domain with a zinc finger motif at N-terminus followed by a Dbl homology domain and Pleckstrin homology domain and a coiled-coil domain at the C-terminus. Inactive GEF-H1 is localized in microtubules, followed by microtubule depolymerization, GEF-H1 is activated and induces its GEF activity towards downstream effector RhoA.
Abbreviations: C1, protein kinase C conserved region 1; GEF-H1, guanine nucleotide exchange factor H1; RHO, ras homolog; GTP, guanosine trisphosphate; GDP, guanosine diphosphate.
Figure is generated based on the information from (Birkenfeld et al., 2008; Joo and Olson, 2021).

1.4.1.1.1.2 Rho GEFs and B lymphopoiesis

The number of human Rho GEFs greatly exceeds the actual number of the Rho GTPases indicating redundancy in Rho GEFs function. RhoA, a prominent member of Rho subfamily of Rho GTPase is activated by at least 28 Dbl Rho GEFs (Cook et al., 2004). Rho GEFs were initially identified as oncoproteins (Eva et al., 1988; Hart et al., 1991). Several studies have shown that Rho GEFs play a critical role in lymphopoiesis as shown in Table 1.

Table 1: Role of Rho GEFs in murine B cell development

	Expression	Knockout phenotype	Reference
Dbl protein			
Vav1	Hematopoietic lineage	Partial defect in T- cell development, normal B-lymphocyte development and function	(Fischer et al., 1998; Turner et al., 1997b).
Vav2	Widely expressed	Normal T and B-cell development. Combined vav1 and vav2 deficiency leads to significant reduction in mature B cells	(Doody et al., 2001; Tedford et al., 2001).
Vav3	Widely expressed, highest levels in hematopoietic cells and brain	Normal lymphocyte development. Mice lacking all three Vav family proteins showed no functional B or T cells.	(Fujikawa et al., 2003)
Lsc/p115 RhoGEF	Hematopoietic lineage	Altered marginal zone B cells and defective immune response to T cell dependent and independent antigens.	(Girkontaite et al., 2001).
Dock protein			
DOCK2	Hematopoietic lineage	Normal B cell development in bone marrow with reduction in transitional and follicular B cells in spleen.	(Ushijima et al., 2018)
DOCK8	Widely expressed	B cells in DOCK8 mutant mice do not develop into MZ B cells or persist in germinal centre and undergo affinity maturation	(Jabara et al., 2012; Randall et al., 2009).
DOCK10	Widely expressed in lymphocytes	Marginal and follicular B cells are reduced in the spleen and peripheral blood of DOCK 10 knock out mice	(García-Serna et al., 2016; Gerasimcik et al., 2017).
DOCK11	Widely expressed in lymphocytes	DOCK11 knock out mice showed increased percentage of early bone marrow cells along with reduced MZ B cells.	(Matsuda et al., 2015).

1.4.1.1.2 GTPase activating proteins (GAPs)

GTPase activating proteins (GAPs) increase the intrinsic rate of hydrolysis of bound GTP thereby inactivating Rho GTPase upon external cues (Van Aelst and D'Souza-Schorey, 1997; Cook et al., 2014). More than 70 members of Rho GAPs have been discovered in eukaryotes (Garrett et al., 1989). Like Rho GEFs, GAPs also outnumber the Rho GTPases which they regulate (Tcherkezian et al., 2005). ARHGAP25 is a hematopoietic specific Rac GAP whose expression is shown to be important for B cell development (Wang et al., 2016). Mice lacking the *Arhgap25* gene have a significant reduction in splenic transitional, follicular and marginal zone B cells along with peripheral blood B cells. The germinal centre formation, isotype switching and plasma cell differentiation are also found to be impaired in *Arhgap25* deficient mice (Lindner et al., 2020).

1.4.1.1.3 Guanine nucleotide dissociation factors (GDIs)

Guanine nucleotide dissociation factors seize Rho GTPase in their inactive GDP-bound state in the cytosol by masking their lipid modification (Van Aelst and D'Souza-Schorey, 1997; Tcherkezian et al., 2005). In mammals, three members of GDIs are identified for the Rho family of GTPases namely GDIα, GDIβ, and GDIγ (Dovas and Couchman, 2005). RhoGDIα is the most abundant and ubiquitously expressed member whereas Rho GDIβ expression is restricted to hematopoietic tissues mainly in T and B lymphocytes. Rho GDIγ expression is preferably found in the kidney, lungs, brain, and pancreas (Adra et al., 1997; Olofsson, 1999; Sasaki and Takai, 1998).

1.4.2 Rho GTPases and B lymphopoiesis

Lymphocytes require Rho GTPase for their normal development and function (He and Westerberg, 2019; Mulloy et al., 2010). The significance of Rho GTPase in lymphopoiesis was first elucidated by overexpressing dominant negative or constitutively active mutants in clonal cell lines (Van Aelst and D'Souza-Schorey, 1997; Gomez et al., 2001). Later using transgenic mice, a crucial role of Rho GTPases in B lymphopoiesis was uncovered (He and Westerberg, 2019; Tybulewicz and Henderson, 2009). Most extensively characterized Rho GTPase subfamilies include Rho, Rac and Cdc42 (Mackay and Hall, 1998). Their role in B cell development and function are listed in Table 2.

Table 2: Rho family GTPases and their role in B cell development

	Gene	Knock out strategy	Phenotype	Reference
Rho	RhoA	Conventional knock out	Embryonic lethal	(Pedersen et al., 2012).
		Mx Cre (hematopoietic stem cells)	Impaired B cell development at CLP differentiation stage	(Zhang et al., 2012).
		CD19 Cre (B cells)	Defective B cell development in spleen with impaired survival response	(Zhang et al., 2012).
	RhoB	Conventional knock out	Normal B lymphopoiesis	(Liu et al., 2001).
	RhoC	Conventional knock out	Normal B cell development	(Hakem et al., 2005).
Cdc42	Cdc42	Conventional knock out	Embryonic lethal	(Chen et al., 2000).
		CD19 Cre (B cells)	Significant reduction in peripheral B cell population and antigen specific immunoglobulin production	(Burbage et al., 2015; Gerasimcik et al., 2015; Guo et al., 2009).
	TC10	mb1- Cre	Normal B-cell development, with defective IgM response upon immunization.	(Burbage et al., 2017).
Rac	Rac1	CD19 Cre (B cells)	Normal B-cell development	(Walmsley et al., 2003).
	Rac2	Conventional knock out	Reduced MZ-B cells and B1-a B cells in the periphery along with reduced Ca^{2+} signalling	(Croker et al., 2002).
	Rac/Rac2	Double knock out (CD19 Cre)	Impaired B cell development at transitional stage in the spleen	(Henderson et al., 2010; Walmsley et al., 2003).

1.4.2.1 Rho subfamily

Rho was first identified and cloned as Ras homolog in 1985 (Madaule and Axel, 1985). It is one of the best characterized subfamilies of Rho GTPases including prototypical family member Rho A and its isoforms RhoB and RhoC (Pedersen et al., 2012). They are small proteins

of 21 kDa exerting their function by binding to downstream effector molecules (Ishizaki et al., 1996). Rho subfamily GTPases are ubiquitously expressed but their sub cellular localization differs. RhoA and RhoC are cytosolic whereas RhoB is found at the plasma membrane and intercellular membrane of vesicles (Pedersen et al., 2012; Wennerberg et al., 2005). Rho subfamily GTPases members are shown to be critical for many fundamental cellular processes like actin cytoskeleton rearrangement, cell migration and cytokinesis (I et al., 1993; Kishi et al.;1993; Ridley and Hall, 1992).

1.4.2.1.1 RhoA

Ras homolog gene family member A (RhoA) is an intracellular signal transducing protein of the Rho family of small GTPases (Van Aelst and D'Souza-Schorey, 1997; Hodge and Ridley, 2016). Upon activation by GEFs RhoA binds to downstream effector molecules such as RhoA-associated coiled coiled kinases1/2 (Rock 1/2) and modulates many cellular events (Amano et al., 1996a; Ishizaki et al., 1996). In vitro studies using constitutively active and dominant negative RhoA mutants together with inhibitory studies utilizing botulinum C3 exoenzyme, a specific inhibitor for Rho elucidated its essential role in cytokinesis, actin stress fibre formation, vesicle trafficking, transcription and cell migration (Raftopoulou and Hall, 2004; Ridley and Hall, 1992).

Structurally RhoA has a core G Domain, followed by an insert region and a hypervariable C terminal domain (Schaefer et al., 2014). G domain is the hallmark of Rho GTPases which is characterized by 5 conserved sequence motifs G1-G5 that mediate guanine nucleotide-binding (Wittinghofer and Vetter, 2011). Switch regions including switch 1 and switch 11 are found within the G domain which is involved in Rho GEF and effector binding (Schaefer et al., 2014; Wittinghofer and Vetter, 2011). Another feature is the Rho insert region located between the G4 and G5 motif, which is primarily involved in the activation and binding of effector molecules (Wennerberg et al., 2005). The C terminus of Rho GTPase comprises a short hypervariable region and CAAX box, which has a lipid anchor that translocate GTPase to the membrane (Figure 8). Hypervariable region functions as a protein binding site that determines the specific binding of GTPase to regulatory or effector proteins (Klooster and Hordijk, 2007; Wennerberg et al., 2005).

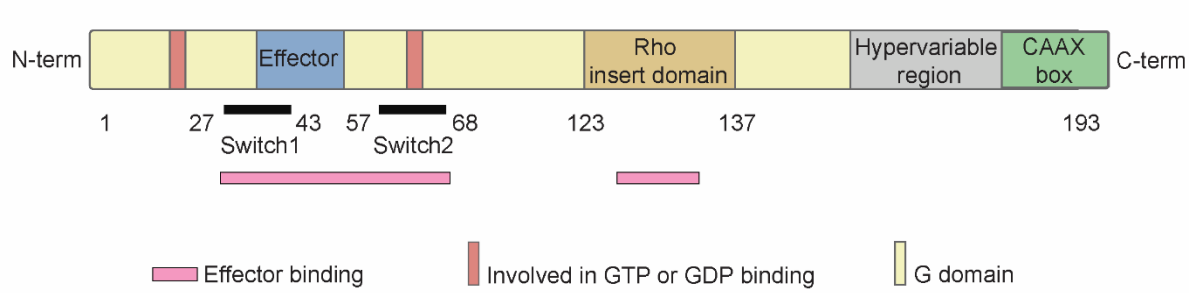

Figure 8: Structure of RhoA: RhoA consists of an N terminal G domain which includes an insert region and is involved in Rho GEF and effectors binding. The C-terminal of RhoA has a short hypervariable region and CAAX box.
Figure is generated based on the information from (Schaefer et al., 2014).

1.4.2.1.2 RhoB and RhoC

Ras homolog gene family member B (RhoB) share 85% sequence similarity with other Rho subfamily GTPases. It was identified and cloned at the same time as RhoA and RhoC (Madaule and Axel, 1985; Thumkeo et al., 2013). RhoB has been shown to be important for vesicle trafficking, endocytosis, antigen presentation in dendritic cells and apoptosis of transformed cells (Fernandez-Borja et al., 2005; Kamon et al., 2006). RhoC isoform of Rho subfamily of GTPases is found overexpressed in many solid cancers and reported to be specifically involved in metastasis (Hakem et al., 2005; Srivastava et al., 2009).

1.4.3 Rho GTPase effector molecules

Upon activation by GEFs, Rho subfamily GTPases bind to a range of effector molecules and modulate their activity and localization (Thumkeo et al., 2013). Rho effectors are predominantly protein kinases which phosphorylate their targets and control cellular functions. Other effectors include scaffold proteins which control cellular functions through protein-protein interactions (Sahai and Marshall, 2002). Main effectors of Rho subfamily kinases include Rho-associated coiled-coil containing kinases (ROCKs), the mammalian homolog of Diaphanous (mDia), Citron, Protein kinase N (PKN), Rhophilin and Rhotekin.

1.4.3.1 ROCKs

Rho-associated coiled-coil containing kinase (ROCK or Rho Kinase) which consists of two mammalian isoforms ROCK1 and ROCK2 is one of the most important Rho effectors (Amano et al., 2010; Nakagawa et al., 1996). ROCKs are ubiquitously expressed serine-threonine kinases playing an important role in regulating actomyosin cytoskeleton rearrangement downstream of RhoA and RhoC (Amano et al., 1996; Julian and Olson, 2014). ROCK proteins are predominantly localized in the cytosol and are activated by Rho-dependent and independent manner (Julian and Olson, 2014; Leung et al., 1995). The physiological roles of ROCKS are elucidated by using small molecule inhibitors in various cell types and animal models. Fasudil (HA-1077), H1152, and Y-27632 are the broadly used Rho kinase selective inhibitors which function in an ATP competitive manner (Shimokawa and Rashid, 2007). Global ROCK knock-out mice generated using gene deletion strategy revealed the role of ROCK proteins in development. ROCK1 and 2 knock-out mice show embryonic lethality after the blastocyst stage (Kamijo et al., 2011; Shimizu et al., 2005). B cell-specific ablation of *Rock2* leads to impaired GC formation, maintenance and defective humoral immune response (Ricker et al., 2020).

1.4.3.2 Other Rho effectors

The mammalian homologue of Drosophila Diaphanous (mDia) was identified as a Rho effector that cooperatively works with ROCK to regulate actin stress fibre formation (Thumkeo et al., 2013; Watanabe et al., 1997). Studies using mDia1 knock-out mice revealed its role in T cell chemotaxis and migration (Eisenmann et al., 2007; Sakata et al., 2007). Citron, a cytokinesis regulating rho effector, has two isoforms: Citron-K, a longer isoform with a kinase domain and found in proliferating cells whereas Citron-N, a smaller isoform without a kinase domain (Di Cunto et al., 1998; Madaule et al., 1998). Protein kinase N (PKN) is activated by binding to Rho and is involved in many cellular processes like cell migration, cell adhesion, and apoptosis (Lachmann et al., 2011; Takahashi et al., 1998). PKN1, an isoform of PKN, knock-out mice

show an enhanced germinal centre formation and develop autoimmune symptoms at an age of 30 weeks. B cells that are deficient for *Pkn1* show increased AKT phosphorylation upon BCR stimulation (Yasui et al., 2012). Rho effector, Rhophilin has two isoforms in mammals whose functions remain relatively unknown (Nakamura et al., 1999; Peck et al., 2002). Rhotekin is a Rho effector that has a high affinity for Rho GTP and is usually used in the pull down assays for activated RhoA (Ren et al., 1999).

1.5 B-cell precursor acute lymphoblastic leukaemia (BCP-ALL)

Leukaemia is the cancer of blood and bone marrow. There are four major types of leukaemia: acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML), acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL). Acute lymphoblastic leukaemia is the malignant transformation and proliferation of lymphoid progenitors, which is found both in adults and children. B-cell precursor acute lymphoblastic leukaemia (BCP-ALL), is the most common type of ALL, characterized by the clonal expansion of malignant B-cell precursors (Margarida Gomes et al., 2014; Terwilliger and Abdul-Hay, 2017). The hallmark of BCP-ALL is chromosomal translocations (such as *E2A-PBX1*, *BCR-ABL1*, *ETV6-RUNX1*) and genetic alterations that occur to the key transcription factors involved in B cell development including IKAROS family zinc finger 1 (*IKZF1*), transcription factor 3 (*E2A*), early B-cell factor (*EBF1*) and paired box 5 (*PAX5*) (Klein et al., 2005). Based on genetic abnormalities B-ALL is classified into different subtypes (Table 3).

Table 3: WHO classification of B-ALL (Terwilliger and Abdul-Hay, 2017).

B-cell lymphoblastic leukemia/lymphoma	Not otherwise specified
B-cell lymphoblastic leukemia/lymphoma	With recurrent genetic abnormalities
B-cell lymphoblastic leukemia/lymphoma	With hypodiploidy
B-cell lymphoblastic leukemia/lymphoma	With hyperdiploidy
B-cell lymphoblastic leukemia/lymphoma	With t(9;22) (q34;q11.2)(*BCR-ABL1*)
B-cell lymphoblastic leukemia/lymphoma	With t(v;11q23) (*MLL* rearranged)
B-cell lymphoblastic leukemia/lymphoma	With t(12;21) (p13;q22)(*ETV-RUNX1*)
B-cell lymphoblastic leukemia/lymphoma	With t(1;19) (q23;p13.3)(*TCF3-PBX1*)
B-cell lymphoblastic leukemia/lymphoma	With t(5;14) (q31;q32)(*IL3-IGH*)
B-cell lymphoblastic leukemia/lymphoma	With intrachromosomal amplification of chromosome 21 (iAMP21)
B-cell lymphoblastic leukemia/lymphoma	With translocations involving tyrosine kinases or cytokine receptors (BCR-ABL1 like ALL)

1.5.1 BCR-ABL1

A prominent BCP-ALL sub-type is Philadelphia chromosome (Ph) positive ALL, which is characterized by the presence of the Breakpoint cluster region-Abelson kinase 1 (BCR-ABL1) fusion protein (Klein et al., 2005). BCR-ABL1 is the product of the Ph chromosome which arises due to the reciprocal translocation between the long arms of chromosomes 9 and 22, t

(9;22) (q34; q11.2) (Hantschel, 2012). Over 90% of chronic myeloid leukaemia (CML), 10-15% of adult ALL and 5% of childhood ALL are characterized by the Ph chromosome (Reichert et al., 2001; Wong and Witte, 2004). There are two isoforms for BCR-ABL1: the p210 isoform is found in CML whereas the expression of the p190 isoform is the characteristic of Ph^+ BCP-ALL (Wong and Witte, 2004). BCR-ABL1 expression is essential for the induction and maintenance of BCP-ALL and interferes with B-lymphoid development by regulating transcription factors and signalling molecules that are necessary for early B-cell development (Klein et al., 2005). BCR-$ABL1^+$ B-ALL cells survive by activating many signalling pathways such as PI3K, MAPK-ERK, Src Kinases, and JAK-STAT (Hantschel, 2012; Klein et al., 2005; Wong and Witte, 2004) which enables them to survive independently of external growth factors (Appelmann et al., 2015; Dorsey et al., 2002; Sillaber et al., 2000). The distinctive feature of BCR-ABL1 oncoprotein is the constitutive activity of the ABL kinase which phosphorylates tyrosine residues located within BCR protein resulting in the activation of numerous signalling pathways (Arlinghaus, 2002). The constitutive tyrosine kinase activity of BCR-ABL1 can be inhibited effectively by small molecule tyrosine kinase inhibitors (TKIs) such as imatinib (Hantschel, 2012). Imatinib is a 2-phenylaminopyrimidine tyrosine kinase inhibitor with an IC_{50} value between 0.1 to 0.5 μM. It binds to the ATP binding site of BCR-ABL1 and stabilizes the inactive non-ATP binding form of BCR-ABL1 thereby preventing tyrosine auto-phosphorylation and subsequent activation of downstream substrates (Deininger et al., 2005). Multiple point mutations in the kinase domain of BCR-ABL1 have been stated as the prime reason for imatinib resistance (Deininger et al., 2005). To treat such patients, second-generation TKIs such as dasatinib and nilotinib are developed, both are approved for the treatment of imatinib intolerant or resistant patients (Soverini et al., 2011; Weisberg et al., 2007).

1.5.2 RhoA-ROCK signalling pathway in leukaemia and lymphoma

RhoA and its downstream targets ROCKs are found deregulated in many hematopoietic and non-hematopoietic malignancies (Benitah et al., 2004; Itoh et al., 1999; Lochhead et al., 2010; Sahai and Marshall, 2002). Hyperactivation of Rho kinases is observed in myeloid leukaemia and myeloproliferative diseases harbouring activating mutations in KIT, FLT3 and BCR-ABL1 and is shown to be dependent on RhoA and PI3K signalling (Singh Mali et al., 2014). On the other hand, inactivating mutations in RhoA and its activators are frequently observed in angioimmnuoblastic and Peripheral T cell lymphomas (Manso et al., 2014; T et al., 2014). Similar inactivating mutations in RhoA and its activator ARHGEF1 are also found in Burkitt's and GC-derived diffuse large B cell lymphomas (O'Hayre et al., 2016). Chronic myeloid leukaemia (CML) harbouring p210 BCR-ABL1 oncogenic fusion protein has a Rho GEF domain which activates RhoA and has been shown to be required for the transforming phenotype (Sahay et al., 2008).

1.6 Aim of the study

B lymphopoiesis is a tightly coordinated developmental process regulated by the lineage-specific cell surface markers, transcription factors and signalling cascades which ultimately lead to the rearrangement of immunoglobulin (Ig) and expression of precursor B-cell antigen receptor (pre-BCR)/BCR on the surface of developing B cells. Pre-BCR/BCR signalling regulates the proliferation, differentiation and selection of B cells at various developmental stages. One of the important signalling pathways activated downstream of BCR engagement is the phosphoinositide 3-Kinase (PI3K) signalling axis, which regulates diverse functions in developing B cells. The adaptor protein, SLP-65, which is also activated upon pre-BCR/BCR activation was shown to induce pre-B cell differentiation by downregulation of PI3K signalling and stabilization of FoxO proteins that activate the recombination machinery.

However, it is not clear how SLP-65 regulates PI3K signalling. Therefore, the main objective of the study is to investigate the mechanism by which SLP-65 modulates PI3K signalling. The main hypothesis is that the small GTPase RhoA, is a novel interaction partner that connects SLP-65 activation with the regulation of PI3K signalling. To better understand the importance of RhoA in B cells, we study its role during B cell development and the generation of proper antibody responses. RhoA and its downstream kinases are found deregulated in some hematopoietic malignancies. Therefore, in this study, we investigate the significance of RhoA and its downstream signalling kinases (ROCKs) in the survival and migration of BCR-ABL1 transformed BCP-ALL cells.

2 Material and methods

2.1 Materials

2.1.1 Equipments- General

	Company
AutoMACS Pro seperator	Miltenyi Biotech
Bacterial incubator	Heraeus B-6120 (50 Hz) Gravity-Convection Incubator, Heraeus instruments
Bacterial shaker incubator	New Brunswick™Innov™44
Balance	Kern EG Series High Accuracy Balance
ELISA plate reader	Multiskan FC, Thermo Fisher Scientific
Flow Cytometer FACS Canto II	BD Biosciences
Flow Cytometer FACS LSR Fortessa	BD Biosciences
Freezer (-80°C)	HERAfreeze™ HFU T series, Thermo Fisher Scientific
	HERAfreeze™ HFU B series, Thermo Fisher Scientific
Freezer (-20°C)	Liebherr-Medline
Filtration systems	Stericup, Milipore Corporation
Gel-documentation system	Heolab Easy RH, ThermoFisher Scientific
Heating blocks	Eppendorf Thermomixer Eppendorf GmbH
Ice flaker machine	Scotsman AF10 Ice Flaker, Scotsman
Nanophotometer	Nanodrop P-class, Implen
Mini-centrifuge	PeqLab Biotechnologie GmbH
Printing unit	Mitsubishi P-93DW Printer, Mitsubishi Electric
Power supply unit	Bio-Rad Powerpac 300, Bio-Rad Laboratories
	Bio-Rad Powerpac 3000, Bio-Rad Laboratories
pH-meter	766 laboratory-ph-meter, KNIK
Refrigerated Multifuge X3R	HeraeusTM, Thermo ScientificTM
Refrigerated Centrifuge	Eppendorf Microcentrifuge 5417 R, Eppendorf GmbH
Refrigerator (4°C)	Liebherr-Medline
Shaker	Heidolph Polymax 1040 Laboratory Platform Shaker
	Heidolph Polymax 1030 Laboratory Platform Shaker
Thermocyclers	C1000 Touch TM Bio-Rad Laboratories
	PEQSTAR 2X Gradient, PEQLAB Biotechnologie, GmbH
	T3 Thermocycler-Biometra
Vortex	Genie 2 Render & Hobin AG

Vaccum System	Mini vacuum pump,13 LPM75 Torr, LaboPort
Voltage equipment	EPS 601, LKB GPS 200/400, EPS 3500, Amersham Pharmacia Biotech
Water bath	GFL, M2218, ProfiLab24
Western blot imaging	Fusion SL, Vilber

2.1.1.1 Cell culture

Equipment	Company
Cryobox	2800 Frigocut, Reichert-Jung
Incubator	Heracell 240
Laminar flow hood	HERAsafe model: HS12, Heraeus instruments
Microscope	Nickon TMS 12441

2.1.1.2 Proximity ligation assay

Equipment	Company
100, 500, 2000 ml Bottles	SHOTT Duran
Centrifuge 5424R	Eppendorf
Diagnostic microscope slides 8 well 6mm numbered	Thermo Fisher Scientific
Duomax 1030 shaker	Heidolph
Gold seal Coverslips 24x50mm 1 1/2H	Electron Microscopy sciences
Grease pen	Science services
High precision coverslips 22x22mm 1.5H	Marienfeld
Leica Microscope:	
CTR Compact	Leica
DFC9000GT Camera	Leica
DMi8 Microscope	Leica
Kübler CODIX Shutter	Leica
SP-box control panel	Dr. Kassen GmbH
TMS Microscope 301309	Nikon
Vacuum Pump Vacu Sip	Integra
Vortex	VWR Chemicals
Warming cupboard	Heraeus

2.1.2 Disposables

Materials	Company
Adhesive qPCR seal	Sarstedt AG & Co
15, 50 ml Blue-cap tubes	Greiner Bio-one GmbH
Cell strainer 100 µM, 40 µM	BD Biosciences

Disposable insulin needle 100 Sterican 26 G x ½'' 0.45 x 12 mm	Braun
Disposable insulin needle 100 Sterican 22 G x ½'' 0.45 x 12 mm	Braun
5 ml round bottom FACS tubes	Sarstedt AG & Co
5 ml round bottom FACS tubes with cell-strainer cap	Corning Falcon
Filter tips 20 µl, Biosphere	Sarstedt AG & Co
Filter tips 20 µl, 200 µl, 1000 µl	Greiner Bio-one GmbH
Immobilion-P membrane, PVD	Merck, Millipore
5, 10 ml syringes Luer lock	BD Biosciences
1.5, 2 ml Micro tubes safe seal	Sarstedt AG & Co
Microscope slides with frosted end ISO 8037/1, 76 x 26 x 1 mm	Menzel
Microtainer	BD Biosciences
96-well Maxisorp ELISA plates	Nunc
2 ml pipettes	Sarstedt AG & Co
5, 10, 25 ml pipettes	Greiner Bio-one GmbH
Pipet tips 0.5-20 µl	Eppendorf
Pipet tips 2-200 µl	Eppendorf
Pipet tips 200-1000 µl	Eppendorf
PCR strips of 8 tubes	BRAND
PCT lids of 8 tubes	BRAND
96 Fast PCR-Plate half skirt	Sarstedt AG & Co
Single use syringes 2, 5,10 ml	Braun
Whatman FP30/0.2 Filter	GE healthcare
Whatman FP30/0.45 Filter	GE healthcare

2.1.2.1 Cell culture

Materials	Company
Cryotubes 2 ml, round bottom	Greiner Bio-one GmbH
KOVA® System counting chamber	Hycor Biomedical
Petri dishes 10, 20, 30 mm	Thermo Fisher Scientific
20 mm culture dishes with grid	Corning Falcon
20 mm culture dishes without grid	Corning Falcon
96-well MicroWell™ plates: Flat (F), Round (R), and Conical (V) bottom	Thermo Fisher Scientific
6, 12- well cell culture plates	Costar
6, 24- well culture plates	Greiner Bio-one GmbH

2.1.3 Chemicals

Reagent	Company
Acetic acid	Prolabo
Agarose	Lonza
Ammonium chloride (NH_4Cl)	Carl Roth GmbH
Ammonium peroxodisulfate (APS)	Sigma-Aldrich
Ampicillin	Anreae-Noris-Zahn-AG
7-Amino-actinomycin D (7-AAD)	Invitrogen
ß-Mercaptoethanol	Gibco life Technologies
ß -Glycerophospahte	Sigma-Aldrich
BSA (fraction V)	Serva
Bradford Protein Assay Solution	BioRad Laboratories
Bromophenol blue	Merck
Diethanolamine solution	Merck
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
ECL-Ultra	Lumingen
Ethanol	J.T.Baker
Ethidium bromide (10 mg/ml)	Sigma-Aldrich
Ethylene diamine tetra acetic acid (EDTA)	Applichem
Glycerol	J.T.Baker
Glycine	J.T.Baker
Heparin	Ratiopharm
Hydrochloric acid (HCl), fuming 37%	Merck
Isopropanol	Sigma-Aldrich
NIP(7)-BSA	Biosearch Technologies Inc.
Methanol	J.T.Baker
$MgCl_2.6H_2O$	VWR chemicals
Protein ladder	Fermentas
Protease Inhibitor Cocktail	Sigma-Aldrich
pNPP tablets-20 mg	Genaxxon bioscience
Rotiphorese®NF-Acrylamide/Bis-solution 30	Carl Roth GmbH
Saponin	Carl Roth GmbH
Sodium Azide (NaN_3)	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Serva
Sodium hydroxide (NaOH) 1 M	Fluka
Sodium Chloride (NaCl)	Fluka
Sodium fluoride (NaF)	VWR chemicals
Sodium orthovanadate (Na_3NO_4)	Applichem
Stripping buffer	Thermo Fisher Scientific
Tetramethyethylendiamine (TEMED)	Carl Roth GmbH
Triton X-100	Merck
Tris-Base	Sigma-Aldrich

Tween-20	Sigma-Aldrich

2.1.4 Reagents

2.1.4.1 Molecular biology

Reagent	Company
Calf intestinal phosphatase (CIP)*	Roche Diagnostics
Dream Taq DNA-Polymerase*	Thermo Fisher Scientific
Dithiothreitol solution (DTT)	Sigma-Aldrich
dNTPs (25 mM)	Genaxxon bioscience
GeneRuler 100 bp plus DNA ladder	Invitrogen
GeneRuler 1 kb DNA ladder	Invitrogen
$MgCl_2$ (25 mM)	Genaxxon bioscience
Oligo $d(T)_{18}$ primer	Fermentas
Proteinase K	Genaxxon bioscience
Q5 High-Fidelity DNA Polymerase*	New England Biolabs
Rediload	Invitrogen
Restriction endonucleases*	New England Biolabs
RevertAid Reverse Transcriptase*	Fermentas
TaqMan-Gene Expression Master mix	Applied Biosystems
T4 DNA-Ligase*	Fermentas

*All enzymatic reactions were performed in buffers provided by the respective company.

2.1.4.2 Dyes

Reagent	Company
Gel loading dye (6X)	New England Biolabs
Propidium Iodide	Invitrogen

2.1.4.3 Cell culture

Reagent	Company
AMD3100	Sigma-Aldrich
C-T04, RhoA inhibitor	Cytoskeleton
Dasatinib	Selleck chemicals
DMSO	Sigma-Aldrich
Dulbecco's phosphate buffered saline(PBS)	Gibco lifetechnologies
Doxycycline	Sigma-Aldrich
FCS	PAN Biotech, Sigma
Genejuice™ Transfection reagent	Merck Millipore
Imatinib	Selleck chemicals
Indo-1-AM	Invitrogen
Iscove's basal medium w/o L-Glutamine	Biochrom (Merck)
4-hydroxytamoxifen (4-OHT)	Sigma-Aldrich

L-Glutamine (200 mM)	Gibco lifetechnologies
Lipopolysaccharides from *Escherichia coli* O111:B4	Sigma-Aldrich
2-Mercaptoethanol (50 mM)	Gibco lifetechnologies
Murine recombinant IL-7	R & D Systems
Murine recombinant CXCR4	R & D Systems
Penicillin /Streptomycin (10000 U/ml)	Gibco lifetechnologies
Pluronic F-127	Invitrogen
Protease inhibitor cocktail	Sigma-Aldrich
Polybrene	Sigma-Aldrich
Puromycin	InvivoGen
RPMI media 1640	Gibco lifetechnologies
Trypan blue solution 4%	Sigma-Aldrich
Trypsin-EDTA solution (0.5%)	Gibco lifetechnologies

2.1.4.4 Proximity ligation assay

Reagent	Company
5X Amplification buffer	Sigma Duolink
Antibody diluent	Sigma Duolink
Blocking solution	Sigma Duolink
Flouromount-G with DAPITM	eBIoscience
Ligase	Sigma Duolink
5X Ligation buffer	Sigma Duolink
Paraformaldehyde (4%)	Serva
PLA Probe anti-mouse +and -	Sigma Duolink
PLA Probe anti-rabbit + and -	Sigma Duolink
PLA Probe maker	Sigma Duolink
Polymerase	Sigma Duolink
Triton X-100	Merck

2.1.5 Media and buffers for cell culture

2.1.5.1 FACS buffer: PBS+5% FCS

Components	Volume/Percentage
FCS	5%
Fill up the required volume using 1X PBS	

2.1.5.2 Iscove's medium with 1% FCS for Ca2+ measurement

Components	Volume/Percentage
FCS	1%

Fill up the required volume using Iscove's basal medium

2.1.5.3 PBS-Trypsin (0.05%)

Components	Volume/Percentage
0.5% Trypsin (10X)	0.05%
Fill up the required volume using 1X PBS	

2.1.5.4 Freezing medium

Components	Volume/Percentage
DMSO	10% (v/v)
Fill up the required volume using FCS	

2.1.5.5 Erythrocyte lysis buffer

Components	Amount (g)
NH_4Cl	8,29
$KHCO_3$	1
EDTA-Na_2	0.0372
Fill up tp 1000 ml with H_2O and adjust pH to 7.2 -7.4	

2.1.5.6 0.5% Saponin Buffer

Components	Amount (g)
Saponin	0.5
Sodium azide	0.02
Fill up to 100 ml using 1X PBS	

2.1.5.7 Iscove's medium+10% FCS+IL-7

Components	Volume/Percentage
FCS	10% (v/v)
Iscove's basal medium	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml
2-Mercaptoethanol (50 mM)	0.5 ml
IL-7 Supernatant	5 ml

2.1.5.8 Iscove's medium+5% FCS+IL-7

Components	Volume/Percentage
FCS	5% (v/v)
Iscove's basal medium	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml
2-Mercaptoethanol (50 mM)	0.5 ml
IL-7 Supernatant	6 ml

2.1.5.9 Iscove's medium+10% FCS-IL-7

Components	Volume/Percentage
Iscove's basal medium	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml
2-Mercaptoethanol (50 mM)	0.5 ml
IL-7 Supernatant	5 ml

2.1.5.10 RPMI medium+5 %FCS

Components	Volume/Percentage
FCS	5% (v/v)
RPMI 1640	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml
2-Mercaptoethanol (50 mM)	0.5 ml

2.1.5.11 RPMI medium+10%FCS

Components	Volume/Percentage
FCS	10% (v/v)
RPMI 1640	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml
2-Mercaptoethanol (50 mM)	0.5 ml

2.1.5.12 RPMI medium+10%FCS+ 2,5 µM Doxycycline

Components	Volume/Percentage
FCS	10% (v/v)
RPMI 1640	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml

2-Mercaptoethanol (50 mM)	0.5 ml
Doxycycline (2,5 µM)	1.5 ml

2.1.5.13 McCoy's medium+20% FCS

Components	Volume/Percentage
FCS	20% (v/v)
McCoy's medium	500 ml
L-gluatmine (10 mM)	5 ml
Pencillin/Streptomycin (100 U/ml)	5 ml
2-Mercaptoethanol (50 mM)	0.5 ml
IL-7 Supernatant	5 ml

2.1.6 Solutions and buffers

2.1.6.1 Genomic DNA extraction

2.1.6.1.1 1 M Tris solution, pH 8.5

Components	Amount (g)
Tris-Base	121.1
Fill up to 800 ml with H_20, adjust pH to 8.5 and fill upto1000 ml with H_20	

2.1.6.1.2 0.5 M EDTA

Components	Amount (g)
EDTA	186.1
Fill up to 800 ml with H_20, adjust pH to 8 and fill upto1000 ml with H_20	

2.1.6.1.3 10% SDS Solution

Components	Amount (g)
SDS	10
Fill up to 80 ml with H_20, adjust pH to 7.2 and fill upto100 ml with H_20	

2.1.6.1.4 5 M NaCl Solution

Components	Amount (g)
NaCl	29.22
Fill up to 100 ml with H_20	

2.1.6.1.5 1 M Tris Solution, pH 8.0

Components	Amount (g)
Tris-Base	121.1
Fill up to 800 ml with H_20, adjust pH to 8.0 and fill upto1000 ml with H_20	

2.1.6.1.6 Lysis buffer

Components	Volume/Percentage
Tris solution (1 M, pH 8.5)	50 ml
EDTA solution (5 M, pH 8.0)	5 ml
SDS Solution (10%)	10l
NaCl Solution (5 M)	20 ml
Fill up to 500l with H_20 and autoclave prior to use	

2.1.6.1.7 TE buffer

Components	Volume/Percentage
Tris solution (1 M, pH 8.0)	1 ml
EDTA solution (5 M, pH 8.0)	200 µl
Fill up to 500l with H_20 and autoclave prior to use	

2.1.6.1.8 Lysis buffer working solution

Components	Volume/Percentage
Lysis buffer	500 ml
Proteinase K	2.5 µl

2.1.6.2 Immunoblotting

2.1.6.2.1 4X Separation buffer

Components	Amount (g)
Tris-Base	181.7
SDS	4
Fill up to 800 ml with H_20, adjust pH to 8.8 and fill upto1000 ml with H_20	

2.1.6.2.2 4X Stacking buffer

Components	Amount (g)
Tris-Base	60.6
SDS	4
Fill up to 800 ml with H_20, adjust pH to 6.8 and fill upto1000 ml with H_20	

2.1.6.2.3 10X Running buffer

Components	Amount (g)
Tris-Base	30.3
Glycine	141.1
SDS	10
Fill up to 800 ml with H_20, adjust pH to 8.6 and fill upto1000 ml with H_20	

2.1.6.2.4 10X Transfer buffer

Components	Amount (g)
Tris-Base	30.3
Glycine	141.1
Fill up to 800 ml with H_20, adjust pH to 8.6 and fill upto1000 ml with H_20	

2.1.6.2.5 1 M Tris solution, pH 7.4

Components	Amount (g)
Tris-Base	121.1
Fill up to 800 ml with H_20, adjust pH to 7.4 and fill upto1000 ml with H_20	

2.1.6.2.6 10X TBS buffer

Components	Amount (g)/Volume (ml)
Tris solution (1 M pH 7.4)	500
NaCl	90
Fill up to 1000 ml with H_20	

2.1.6.2.7 Washing buffer

Components	Volume/Percentage
10X TBS buffer	100 ml
Tween-20	1 ml
Fill up to 1000 ml with H_20	

2.1.6.2.8 Blocking buffer

Components	Amount (g)
BSA	2.5
Fill up to 50 ml mark in 50 ml falcon tube with washing buffer	

2.1.6.2.9 10% SDS solution

Components	Amount (g)
SDS	10
Fill up to 100 ml with H_20	

2.1.6.2.10 0.5 M EDTA solution

Components	Amount (g)
EDTA	186.1
Fill up to 800 ml with H_20, adjust pH to 8.0 and fill upto1000 ml with H_20	

2.1.6.2.11 0.1% Bromophenol blue

Components	Amount (g)
Bromophenol blue	0.1
Fill up to 10 ml with H_20	

2.1.6.2.12 10% Ammoniumpersulfate solution

Components	Amount (g)
Ammoiumpersulfate	1
Fill up to 10 ml with H_20	

2.1.6.2.13 RIPA buffer

Components	Amount (g)/Volume (ml)
1% Triton X-100 solution	2 ml
SDS Solution (10%)	10 ml
Tris solution (1 M, pH 6.8)	10 ml
NaCl	1.64 g
Fill up to 200 ml with H_20 and adjust pH to 8 and filter prior to use	

2.1.6.2.14 5 mM NAF solution

Components	Amount (g)
NaF	20.5
Fill up to 100 ml with H_20	

2.1.6.2.15 50 mM 2-Glycerophosphate

Components	Amount (g)
ß-Glycerophosphate	1.08
Fill up to 100 ml with H_20	

2.1.6.2.16 1 mM Sodium orthovanadate

Components	Amount (g)
Sodium orthovanadate	18,39
Fill up to 100 ml with H_20	

2.1.6.2.17 RIPA++ buffer

Components	Volume/Percentage
RIPA buffer	830 µl
Protease inhibitor cocktail	100 µl
NaF (5 mM)	10 µl
ß-Glycerophosphate (50 mM)	50 µl
Sodium orthovanadate (1 mM)	10 µl

2.1.6.2.18 Lilly's 4X sample buffer

Components	Volume/Percentage
SDS solution (10%)	20 ml
Tris (1 M, pH 6.8)	10 ml
Glycerol	10 ml
0.1% Bromophenolblue	2.5 ml
Fill up to 50 ml with H_20 and add 5% ß-mercaptoethanol prior to use	

2.1.6.2.19 10% SDS separating gel

Components	Volume/Percentage
H_20	3 ml
Separating buffer	2 ml
Acrylamide/Bis	2.7 ml
APS (10%)	75 µl
TEMED	7.5 µl

2.1.6.2.20 4% stacking gel

Components	Volume/Percentage
H_20	1.8 ml
Stacking buffer	0.9 ml
Acrylamide/Bis	0.4 ml
APS (10%)	30 µl
TEMED	3 µl

2.1.6.3 Co-Immuoprecipitation

2.1.6.3.1 250 mM Tris solution, pH 7.5

Components	Amount (g)
Tris-base	7.6
Fill up to 200 ml with H_20, adjust pH to 7,5 and fill up to 250 ml	

2.1.6.3.2 500 mM NaCl solution

Components	Amount (g)
NaCl	7.3
Fill up to 250 ml with H_20	

2.1.6.3.3 100 mM EDTA, pH 8

Components	Amount (g)
EDTA	1,86
Fill up to 40 ml with H_20, adjust pH to 8 and fill up to 50 ml	

2.1.6.3.4 Co-immunoprecipitation lysis buffer

Components	Volume/Percentage
EDTA (100 mM)	2 ml
Tris (250 mM, pH 8)	40 ml
NaCl (500 mM)	60 ml
Triton X-100 (1%)	200 µl
Fill upto 180 ml, adjust pH to 8 and fill up to 200 ml and filter prior to use	

2.1.6.3.5 Co-immunoprecipitation lysis buffer++

Components	Volume/Percentage
Co-immunoprecipitation lysis buffer	830 µl
Protease inhibitor cocktail	100 µl
NaF (5 mM)	10 µl
ß-Glycerophosphate (50 mM)	50 µl
Sodium orthovanadate (1 mM)	10 µl

2.1.6.3.6 Co-immunoprecipitation Wash buffer

Components	Volume/Percentage
EDTA (100 mM)	2 ml
Tris (250 mM, pH 8)	40 ml
NaCl (500 mM)	60 ml
Triton X-100 (1%)	2 ml
Fill upto 180 ml, adjust pH to 8 and fill up to 200 ml	

2.1.6.3.7 Co-immunoprecipitation Wash buffer++

Components	Volume/Percentage
Co-immunoprecipitation wash buffer	830 µl
Protease inhibitor cocktail	100 µl
NaF (5 mM)	10 µl
ß-Glycerophosphate (50 mM)	50 µl
Sodium orthovanadate (1 mM)	10 µl

2.1.6.4 ELISA

2.1.6.4.1 ELISA Washing buffer

Components	Volume/Percentage
PBS (10X)	100 ml
Tween 20	500 µl
NaN_3 (10%)	1 ml
Fill up to 500 ml with H_20	

2.1.6.4.2 ELISA Blocking buffer

Components	Volume/Percentage
BSA	5 g
NaN_3 (10%)	500 µl
Fill up to 500 ml with H_20	

2.1.6.4.3 4 mM $MgCl_2$

Components	Amount (g)
$MgCl_2.6\ H_20$	0.8 g
Fill up to 1000 ml with H_20	

2.1.6.4.4 Diethanolamine buffer

Components	Volume/Percentage
Diethanolamine	97 ml
$MgCl_2$ (4 mM)	500 µl
NaN_3 (10%)	2 ml
Adjust pH to 9.8	

2.1.6.4.5 pNPP substrate solution

Components	Volume/Percentage
Diethanolamine buffer	40 ml
pNPP	1 Tablet

2.1.6.4.6 3 M NaOH solution

Components	Amount (g)
NaOH	58,5 g
Fill up to 500 ml with H_20	

2.1.6.5 Proximity ligation assay

2.1.6.5.1 Wash A

Components	Amount (g)
Nacl (150 mM)	8.8
Tris Base (10 mM)	1.2
Tween 20 (0.05%)	50 ml
Fill up to 800 ml with H_20, adjust pH to 7.4 and fill up to 1000 ml	

2.1.6.5.2 Wash B

Components	Amount (g)
Nacl (100 mM)	5.84
Tris Base (200 mM)	4.24
Tris HCl	26
Tween 20 (0.05%)	50 ml
Fill up to 800 ml with H_20, adjust pH to 7.5 and fill up to 1000 ml	

2.1.7 Media for bacterial culture

2.1.7.1 LB media

Components	Amount (g)
Trypton	10
Yeast extract	5
NaCl	5
Fill up to 800 ml with H_20, adjust pH to 7.5 and fill up to 1000 ml. Autoclave prior to use	

2.1.7.2 LB-Agar plates

Components	Amount (g)
Agarose	2
Added per 100 ml of LB media.	

2.1.7.3 LB-Amp media

Components	Amount (µg)
Ampicillin	100
Added per ml of LB media.	

2.1.7.4 1M $MgSO_4.7H_20$

Components	Amount (g)
$MgSO_4.7H_20$	246
Fill up to 1000 ml with H_20	

2.1.7.5 1M $MgCl_2.6H20$

Components	Amount (g)
$MgCl_2.6H_20$	203.3
Fill up to 1000 ml with H_20	

2.1.7.6 1M KCl

Components	Amount (g)
KCl	74.55
Fill up to 1000 ml with H_20	

2.1.7.7 5M NaCl

Components	Amount (g)
NaCl	292
Fill up to 1000 ml with H_20	

2.1.7.8 1M $CaCl_2.2H_20$

Components	Amount (g)
$CaCl_2$. $2H_20$	147.02
Fill up to 1000 ml with H_20	

2.1.7.9 250 mM Pipes

Components	Amount (g)
Pipes	17.315
Fill up to 1000 ml with H_20	

2.1.7.10 1 M $MnCl_2.4H_20$

Components	Amount (g)
$MnCl_2.4H_20$	197.92
Fill up to 1000 ml with H_20	

2.1.7.11 SOB medium

Components	Amount (g)/Volume (ml)
Bacto-Tryptone (2%)	20
Yeast extract (0.5%)	5
KCl (I M)	2.5 ml
NaCl (5 M)	2 ml
Fill up to 800 ml with H_20, adjust pH to 7.5 and fill up to 1000 ml. Autoclave prior to use	
MgSO4 (1 M), sterile filtered	10 ml
MgCl2 (1 M), sterile filtered	10 ml

2.1.7.12 SOC medium

Components	Volume/Percentage
Glucose (2 M)	10 ml
Fill up to 1000 ml using SOB medium	

2.1.7.13 TB medium

Components	Volume/Percentage
Pipes (250 mM, pH 6.5)	10 ml
CaCl2 (1M)	3.75 ml
KCl (I M)	62.5 ml
Fill up to 200 ml with H_20, adjust pH to 7.5 and fill up to 250 ml. Autoclave prior to use	

MnCl2 (1 M), sterile filtered	13.75 ml

2.1.8 Bacterial strains

Bacteria	**Genotype**
Escherichia coli DH5α	fhuA2 Δ (argF-lacZ) U169 phoA glnV44 Φ 80 Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17
Epicurian Coli XL10-Gold	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔ M15 Tn 10 (Tetr)]

2.1.9 Retroviral expression vectors used in the study:

Vector	Reporter gene / selectable marker	Reference
pMIG-RhoA	IRES-GFP	Generated in the study
pMIG-BCR ABL	IRES-GFP	(Abdelrasoul et al., 2020)
pMIG-FOXO1	IRES-GFP	(Alkhatib et al., 2012)
pMIG-Pten	IRES-GFP	(Alkhatib et al., 2012)
MSCV-ER^{T2}	Puromycin resistance	(Herzog et al., 2008)
MSCV-Cre ER^{T2}	Puromycin resistance	(Herzog et al., 2008)

2.1.10 Primers used in the study

2.1.10.1 Genotyping and deletion PCR

Allele	**Primer**	**Sequence 5'-3'**	**Expected band Size (bp)**
RhoA$^{fl/fl}$	JVH11	5'-AGC CAG CCT CTT GAC CGA TTT A-3'	RhoA floxed - 393
	JVH14	5'-ATG TCA AAG AGG AAA TAC TGC-3'	RhoA wt -273
Mb1-hcre	hCre-For	5'-ACC TCT GAT GAA GTC AGG AAG AAC-3'	Mb1-hcre- 500
	hCre-Rev	5'-GGA GAT GTC CTT CAC TCT GAT TCT-3'	
Mb1-wt	Mb1-dir	5'-CTG CGG GTA GAA GGG GGT C-3'	Mb1-wt- 400
	Mb1-in2rev	5'-CCT TGC GAG GTC AGG GAG CC-3'	
CD21-Cre	CD21 NDE	5'-TCT GGC ATA CTT ATT CCC TGA AG-3'	CD21 Cre- 450
	Cre rev	5'-GAA CCT CAT CAC TCG TTG CAT C-3'	
Actin	m-Actin-F	5'- GATCACTATTGGCAACGAGC-3'	Actin- 400
	m-Actin-R	5'-ACGCAGCTCAGTAACAGTCC-3'	

RhoA deletion	m-RhoA-F	TGC CAT CAG GAA GAA ACT CGT	RhoA deleted-574
	m-RhoA-R	CAA GAT GAG GCA CCC AGA CTT	

2.1.10.2 Sequencing

Primer	Sequence
MIG++	TAAGCCTCCGCCTCCTCT
MIG--	TCACA TTGCCAAAAGACGG
MIG+	TCC TCC CTT TAT CCA GC

2.1.10.3 Taq-Man primers probes

Taq-Man Probe mixes	Applied Biosystems ID
mGAPDH	Mm99999915_g1
mRAG-1	Mm01270936_m1
mRAG-2	Mm01270938_m1

2.1.10.4 SYBR Green primer probes

SYBR GREEN primers	ID
hGAPDH	Hs_GAPDH_1_SG
hRHOA	Hs_RHOA_1_SG

2.1.11 Cell lines used in the study

2.1.11.1 Murine cell lines

Cell line	**Origin**
$RhoA^{fl/fl}$	Bone marrow derived pre-B cell line
$RhoA^{fl/fl}$ ER^{T2}	Bone marrow derived pre-B cell line
$RhoA^{fl/fl}$ $CreER^{T2}$	Bone marrow derived pre-B cell line
TKO	Bone marrow derived pre-B cell line from $Rag2^{-/-}$ $\lambda5^{-/-}$ $SLP\text{-}65^{-/-}$ reconstituted with expression vector for ER^{T2}-SLP-65 and pre-BCR

2.1.11.2 Human cell lines

Cell line	**Origin**
EU-3	B cell precursor leukaemia cell line established from bone marrow of 12-year-old boy with ALL
TOM-1	B cell precursor leukaemia cell line established from bone marrow of 54-year-old woman with refractory BCR-$ABL1^{+}$ ALL
SUP-B15	B cell precursor leukaemia cell line established from bone marrow of 9-year-old boy with ALL
Ramos	Human Burkitt's lymphoma cell line

DG-75EB (EcoBlast)	Human Burkitt's lymphoma cell line transduced with an ecotropic receptor
DG-75EB-RhoA-HA	DG-75EB retrovirally transduced with RhoA-HA, which enables the expression of RhoA-HA in the presence of 2.5 µM doxycyline
Phoenix-Eco	HEK293T derived retroviral producer cell line

2.1.12 Antibodies

2.1.12.1 Enzyme-linked immunosorbent assay (ELISA)

	Concentration	**Clone**	**Company**
Coating			
α-IgM	10 µg/ml	Polyclonal	Southern Biotech
α-IgG	10 µg/ml	Polyclonal	Southern Biotech
Standard			
Mouse IgM	5 µg/ml	Polyclonal	Southern Biotech
Mouse IgG	1 µg/ml	Polyclonal	Southern Biotech
Detection			
Goat α-mouse IgM		Polyclonal	Southern Biotech
Goat α-mouse IgG		Polyclonal	Southern Biotech

2.1.12.2 Flow cytometry

Antigen	**Specificity**	**Conjugate**	**Clone**	**Company**
B220/CD45R	Mouse/Human	Pe-cy7	RA3-6B2	eBioscience
CD3e	Mouse	Pe-Cy7	145-2c11	eBioscience
CD5	Mouse	PE	53-7.3	eBioscience
CD11b	Mouse	PE	M1/70	eBioscience
CD19	Mouse	PerCP-Cy5.5	ID3	BD Biosciences
CD21/CD35	Mouse	APC	7E9	eBioscience
CD23	Mouse	PE	B3B4	eBioscience
CD25	Mouse	APC	PC61	BD Biosciences
CD43	Mouse	PE or FITC	S7	BD Biosciences
CD117	Mouse	PE	ACK45	BD Biosciences
Kappa	Mouse	PE	Polyclonal	Southern Biotech
IgD	Mouse	APC	11-26	eBioscience
IgD	Mouse	FITC	11-26	Southern Biotech
IgM	Mouse	eF-450	eB121-15-F9	eBioscience
IgM	Mouse	FITC	Polyclonal	Southern Biotech
IgM	Mouse	Pe-Cy7	2/41	eBioscience
CD16/CD32	Mouse		2.4 G2	BD Biosciences

Sytox™ blue	Mouse	eF-450		Life technologies

2.1.12.3 Immunoblotting

Antigen	Origin	Clone	Company
GEF-H1	Rabbit	55B6	Cell signalling Technology
BLNK (2B11)	Mouse	SC-8003	Santa Cruz Biotechnology
BLNK	Rabbit	DP32H	Cell signalling Technology
p-BLNK	Rabbit	E4P2P	Cell signalling Technology
Plcγ2	Mouse	H-160	Santa Cruz Biotechnology
p-plcγ2	Rabbit	Y759	Cell signalling Technology
GAPDH	Rabbit	14C10	Cell signalling Technology
RhoA	Mouse	SC-418	Santa Cruz Biotechnology
RhoA	Rabbit	67B9	Cell signalling Technology
FOXO1	Rabbit	C29H4	Cell signalling Technology
p-FOXO1(s256)	Rabbit	S256	Cell signalling Technology
AKT	Rabbit	67E7	Cell signalling Technology
p-AKT (s473)	Rabbit	D9E	Cell signalling Technology
PTEN	Rabbit	D4.3	Cell signalling Technology
Anti-Mouse IgG	Horse		Cell signalling Technology
Anti-Rabbit IgG	Goat		Cell signalling Technology

2.1.12.4 Proximity ligation assay

Antigen	Origin	Clone	Company
GEF-H1	Rabbit	55B6	Cell signalling Technology
BLNK	Rabbit	DP32H	Cell signalling Technology
p-BLNK	Rabbit	E4P2P	Cell signalling Technology
RhoA	Mouse	SC-418	Santa Cruz Biotechnology

The antibodies for RhoA and BLNK were labeled directly using the Duolink in situ Probemaker plus/minus reagent. GEF-H1 and pSLP-65were detected by the corresponding secondary antibody Duolink in situ PLA Probe Anti-Rabbit plus/minus.

2.1.13 Kits

Kit	Company
APC BrDU flow kit	BD Pharmingen
CloneJET PCR cloning kit	Thermo Fischer Scientific
CD19 Microbeads, Mouse	Miltenyi Biotech
G-LISA Kit	Cytoskeleton, Inc
High-capacity RNA to cDNA kit	Applied Biosystems
RevertAid Reverse Transcriptase kit	Thermo Fischer Scientific
Reliaprep™ RNA miniprep system	Promega, Inc

Picopure™ RNA isolation kit	Arcturus
QIAquick Gel Extraction kit	Qiagen
QIAquick PCR Purification kit	Qiagen
Plasmid midi and maxi kit	Qiagen

2.1.14 Softwares

Software	**Company**
ImageJ 1.52K	Freeware developed by Wayne Rasband, NIH
FlowJo-v10.6.2	TreeStar
Prism version 9	GraphPad
Mendeley	Elsevier
Geneious	Dotmatics
Adobe illustrator CC	Adobe Inc.
MS office	Microsoft
StepOne software v.2.3	Thermo Fisher Scientific

2.2 Methods

2.2.1 Mice

Table 4: Mice strains used in the study

Mouse strain	Reference
$RhoA^{fl/fl}$	(Herzog et al., 2011)
Mb1 Cre	(Hobeika et al., 2006)
CD21 Cre	(Kraus et al., 2004)

2.2.1.1 Generation of $RhoA^{fl/fl}$ mb1 Cre and $RhoA^{fl/fl}$ CD21 Cre mice

Conditionally targeted Homozygous mice for the floxed *RhoA* allele ($RhoA^{fl/fl}$) were generated as previously described (Herzog et al., 2011). For a conditional inactivation of *RhoA* in early B cell development, $RhoA^{fl/fl}$ mice were crossed with mice expressing a variant of *hCre* recombinase under the control of the B cell-specific *mb1* gene promoter (Hobeika et al., 2006). $RhoA^{fl/fl}$ mice were crossed with mice expressing Cre recombinase under the control of CD21 (Kraus et al., 2004) for the conditional deletion of *RhoA* at mature B cell stages.

2.2.1.2 Ethical statement

Mice used in the study were bred and housed under specific pathogen-free (SPF) conditions in the animal facility of Ulm University. All experimental procedures were done according to the guidelines of Regierungspräsidium Tübingen, Baden Württemberg. Male or female mice used in the study were sacrificed and analyzed at the age of 8 -60 weeks.

2.2.1.3 Mouse analysis

Mice were euthanized with carbon dioxide. Under sterile conditions femurs, tibia and spleen were harvested and stored in FACS buffer (PBS+5% FBS). Femurs and tibia were cut at both ends and were flushed with ice-cold FACS buffer to get bone marrow single cell suspensions. Splenic single cells were prepared by grinding the spleen between the ends of two micro slides or by smashing it through the cell strainer with a 5 ml plunger. Erythrocytes were removed by resuspending bone marrow and splenic cell pellets with 500 µl erythrocyte lysis buffer followed by 5 min incubation at room temperature. Adding 5 ml FACS buffer stopped the lysis reaction. Erythrocytes were removed by centrifugation and bone marrow or splenic cells were resuspended in FACS buffer and stored on ice until further use. Cell numbers were determined from individual organs before using them for flow cytometric analysis or PCR. Tail tips were stored to confirm the genotype of the analyzed mice. Blood from living mice was collected by a transverse incision through the major tail vein and incubated in a blood collection tube containing heparin, the samples were centrifuged for 5 min at 10000 xg at 4°C. The clear phase containing the serum was transferred into a fresh tube and stored at -80°C till further use.

2.2.1.4 Immunization

$RhoA^{fl/fl}$ (N=7) and $RhoA^{fl/fl}$ $CD21^{+/Cre}$ (N=8) mice of 6-8 weeks of age were immunized with 100 µg NP(24)KLH (Biosearch Technologies California, N-5060-5) + 100µg CpG-ODN1826 (Biomer, custom design) in PBS or with 100µg CpG-ODN1826 only (control immunization).

Sera were collected on days 7, 14 and 21 post-primary immunization (day 0) and after 7 and 35 days after booster immunization (day 21). The serum titre of anti-NP IgM and anti-NP IgG antibodies were measured by sandwich ELISA using NP-BSA (Biosearch Technologies California, N-5050L-10) as coating material and concentrations in arbitrary units (AU) were determined according to a standard (Southern Biotech). The decline of anti-NP-IgG titers was calculated as the ratio of IgG titers at day 28 relative to day 56.

2.2.2 Cell culture

2.2.2.1 Freezing and thawing of eukaryotic cells

For freezing, cells from a dense culture were harvested by centrifugation at 1200 rpm at 4°C for 5 min. The supernatant was aspirated and the pellet was resuspended in a freezing medium. Aliquots of 1 ml containing 1-10 x10^6 cells were transferred to cryotubes. These were quickly put on the ice and then frozen at -80°C. For long storage cells were kept in liquid nitrogen.

To thaw cells, a cryotube was defrosted quickly by shaking in a 37°C water bath, cells were transferred to a previously prepared 15 ml Falcon tube containing 5 ml FCS and centrifuged for 5 min at 1200 rpm and 4°C, subsequently, supernatant was aspirated, the cell pellet was resuspended in the appropriate medium and the cells were plated out in 6 well-plates.

2.2.2.2 Production of IL-7

IL-7 is produced in-house as a sterile filtered supernatant of J558L plasmacytoma cells, which are stably transfected with a vector encoding murine IL-7.

2.2.2.3 Generation of IL-7 dependent pro/pre-B cell cultures

For the generation of IL-7-dependent pro/pre-B cell cultures, freshly isolated murine bone marrow was cultured in Iscove's medium containing 10% heat-inactivated FCS and IL-7. IL-7 allows rapid enrichment of early B cells, which eventually become stably growing cell lines that can grow independent of feeder cells. B cell progenitor cell lines are maintained by the addition of IL-7 in the culture medium and IL-7 withdrawal leads to their rapid cell death.

2.2.2.4 Cultivation of BCR-ABL1 transformed cell lines

BCR-ABL1 transformed pre-B cell lines were cultured in Iscove's medium without IL-7 and were split every two days.

2.2.2.5 Cultivation of Triple knock out (TKO) cells

Triple knock out (TKO) pro B cell line (Übelhart et al., 2015) reconstituted with an expression vector for the pre-BCR and inducible form of SLP-65 cells were cultured in Iscove's medium containing 5% heat-inactivated FCS and IL-7.

2.2.2.6 Cultivation of mature splenic cells

Mature splenic cells were cultured in Iscove's medium without IL-7. Cells were treated with 2.5 µg/ml LPS to assess the survival capacity of mature B cells.

2.2.2.7 Cultivation of phoenix cells

Ecotropic phoenix cells were cultured in Iscove's medium containing 10% heat-inactivated FCS without IL-7. At 60-70% confluency cells were detached with PBS-Trypsin and split at a ratio of 1:10.

2.2.2.8 Cultivation of DG75EB/HA-RhoA and DG75EB cells

DG75EB/HA-RhoA cells are derived from the human Burkitt lymphoma cell line DG75 (Benbassats et al., 1977). The cells were stably transfected to express the murine cationic amino acid transporter 1 (*SLC7A1*) to make them susceptible to infection with MMLV-based retrovirus particles (Engels et al., 2014). These modified cells are termed DG75EB. To obtain HA-RhoA-expressing DG75EB cells, we retrovirally infected the cells with pRetroX-OneTet-Pruo (Clontech) encoding HA-tagged RHOA. Upon treatment with Doxycycline, the cells express HA-RHOA. These cells were cultured in RPMI medium containing 10% heat-inactivated FCS (PAN Biotech) and 1 mg/ml Doxycycline (Sigma Aldrich). DG75EB/HA-RhoA cells were cultured in RPMI medium containing 10% heat-inactivated FCS (PAN Biotech) and 1 mg/ml Doxycycline (Sigma Aldrich). DG75EB parental cells were cultured in a similar medium without Doxycycline.

2.2.2.9 Cultivation of human ALL cell lines

Human B cell precursor leukaemia cell lines EU-3 and TOM-1 were cultivated in RPMI medium with 10% FCS, whereas SUP-B15 were cultured in McCoy's 5A medium supplemented with 20% FCS.

2.2.2.10 Cultivation of Ramos cells

Human Burkitt lymphoma cell line Ramos was cultivated in RPMI medium with 5% FCS.

2.2.2.11 Isolation of $CD19^+$ B cells

Bone marrow single-cell suspension was prepared from $RhoA^{fl/fl}$ and $RhoA^{fl/fl}$ $mb1^{+/hcre}$ mice. $CD19^+$ cells were isolated from these suspensions using an AutoMACS Pro separator using CD19 Microbeads (Miltenyi Biotech) based on the positive selection method according to the manufacturer's instructions. Sorted cells were cultured in Iscove's medium containing 10% heat-inactivated FCS and IL-7 until they are used for retroviral transduction.

2.2.2.12 G-LISA assay

Triple knock-out (TKO) pro B cell line reconstituted with an expression vector for the pre-BCR and inducible form of SLP-65 was used for the study. Cells were serum starved for 72 hours and were either stimulated with 1µM 4-hydroxytamoxifen (4-OHT, Sigma Aldrich) or its control EtOH for different time points. After stimulation cells were then snap-frozen in liquid nitrogen and GTP bound RhoA level was measured using GLISA-Kit (Cytoskeleton, Inc) according to the manufacturer's protocol.

2.2.2.13 RhoA Inhibition

Bone marrow cells derived from wild-type mice were cultured in Iscove's medium supplemented with IL-7 for 5 days. Cells were serum starved for 3 hours before treating with different concentrations of Rho Inhibitor I (CT04, Cytoskeleton, Inc). After 16 hours cells were

collected, washed and lysed with RIPA buffer containing Protease and Phosphatase inhibitor cocktail. The amounts of protein in the lysates were determined using the Bradford assay and proteins of interest were detected using immunoblotting.

2.2.2.14 Inhibition of kinase activity

For kinase inhibition, BCR-ABL1 transformed pre-B cells were treated with 2 µM Imatinib and 100 nM Dasatinib for 24 hours. Kinase inhibitor-treated cells were either used for RNA preparation or analysed by flow cytometry.

2.2.2.15 Migration assay

$5x10^5$ cells were cultured on the top chamber of a transwell culture insert (Corning, pore size 5 µm) and were allowed to migrate toward media containing 100 ng/ml CXCL12 (ImmunoTools) for 16 hours. The number of cells in the lower chamber was determined using a haematocytometer.

2.2.2.16 Induction of deletion of floxed alleles using Cre-ER^{T2} system

Stably growing cell lines of *RhoA*$^{fl/fl}$, *IL-7R*$^{fl/fl}$, *CXCR4*$^{fl/fl}$ bm-derived pre-B cells (transformed with BCR-ABL1 or IL-7 dependent) were retrovirally transduced with tamoxifen-inducible Cre-recombinase (Cre-ER^{T2}) or empty control vectors and subsequently selected by addition of puromycin. Cre-recombinase was activated by the addition of 1- 2 µM 4-OHT. As a control, cells were treated with EtOH (solvent of 4-OHT). Deletion of specific genes was analysed 48 or 72 hours after 4-OHT addition by Flow cytometry, PCR or by immunoblotting.

2.2.3 Flow cytometry

2.2.3.1 Surface and intracellular staining

For flow cytometric analysis, cell suspensions were pre-treated with a-CD16/CD32 Fc-Block for 20 min on ice protected from light. Dead cells were excluded using Sytox blue or fixable viability dye.

For staining surface receptors, cells were stained using the antibodies listed in the materials for 20 min on ice in the dark. Cells were washed with FACS buffer and stored on ice protected from light in FACS buffer until they are analyzed using FACS Canto II (BD Bioscience) or FACS LSR Fortessa (BD Bioscience) flow cytometer. If not indicated otherwise, numbers in the dot plots indicate the percentages of cells in the respective gates, and numbers in the histograms indicate the mean fluorescence intensity (MFI).

Intracellular staining was performed after surface staining using Fix and Perm permeabilization kit according to the manufacturer's instructions. In summary, the cells were fixed and permeabilized in 25 µl of solution A for 15 min at room temperature protected from light. Cells were washed with FACS buffer and resuspended in a 25 µl antibody diluted solution B mix. After 20 min of incubation at room temperature in the dark, cells were washed first with 0.5% saponin and later with FACS buffer. Cells were stored in FACS buffer until they are analyzed.

2.2.4 Plasmids and retroviral transduction

Full-length cDNA encoding murine RhoA was ordered from Integrated DNA Technologies. It was amplified using proofreading Taq Polymerase and sub-cloned into pJET vector1.2/Blunt linearized cloning vector-using CloneJET cloning kit according to the manufacturer's instructions. Ligated products were transformed into DH5α or XL10-Gold as previously described. Successfully recombined colonies were analyzed using Colony PCR using the pJET forward and reverse primers as described earlier. The amplified insert was digested using the restriction enzymes flanking the insert and cloned into the retroviral expression vector backbone of pMIG to generate a RhoA-IRES-GFP expression vector. Plasmids for the expression of BCR-ABL1, FOXO1, PTEN and tamoxifen-inducible form of Cre (Cre-ER^{T2}) are described previously (Alkhatib et al., 2012; Herzog et al., 2008). Plasmids were confirmed by sequencing or by digestion with restriction endonucleases

2.2.4.1 Transfection

Viral supernatants were generated using the Phoenix-Eco retroviral producer cell line. Phoenix cells were cultured in Iscove's culture medium + 10% FCS. 24 hours prior to transfection, cells were plated at a density of 0.25x10^6 cells per ml in a well of a 6 well plate. In the following day, medium was aspirated and replaced with 1.5 ml of fresh medium. For transfecting a single well: 3 µl of the transfection reagent GeneJuice was mixed with 100 µl of Iscove's basal medium, followed by 5 min incubation at room temperature. Subsequently 1 µg of plasmid of interest was added, vortexed shortly and incubated for further 15 mins at room temperature. Afterwards the mixture was added drop wise to the phoenix cells. Retroviral supernatant was harvested after 48 hours later and filtered using 0.45 µM filters. Supernatant was used either freshly for transduction or stored at -80° C for further use.

2.2.4.2 Retroviral transduction

To every 1 ml of the filtered supernatant containing retroviral particles used for the transduction were mixed with 1 µl of polybrene (10 mg/ml). For transduction 0.25x10^6 cells were mixed with 0.5-1 ml of supernatant- polybrene mixture and 1centrifuged at 300 xg for 3h at 37°C. Subsequently, the supernatant was aspirated and the cells were resuspended in an appropriate medium with or without cytokines. The transduced cells were then incubated at 37°C, 7.5% CO2 until analysis.

2.2.5 Protein methods

2.2.5.1 Immunoprecipitation

DG75EB/HA-RhoA cells were serum starved for 30 min and stimulated for 0,1,3,5 and 10 min with 10 µg/ml anti-human IgM F(ab)'2 (Southern biotech) at 37°C in a thermomixer. Adding ice-cold PBS stopped the reactions and cells were lysed using co-immunoprecipitation lysis buffer. Lysates were cleared by centrifugation at 13200 rpm for 15 min at 4°C. Supernatant, which has proteins, were collected and transferred to a fresh vial. Protein concentration was determined using Bradford reagent. In summary 1 µl of sample is mixed with 1 ml of Bradford reagent followed by 5 min incubation at room temperature. Afterwards protein concentration was measured using optical density in a nanophotometer (595 nm). The concentration of

protein was calculated based on the standard curve prepared by different concentration of BSA (1 mg/ml) in Bradford reagent. Immunoprecipitation of cell lysates was performed using Protein G Sepharose 4 Fast Flow 50 % gel suspension (GE health care) with an anti-BLNK antibody according to the manufacturer's instructions. Precipitated proteins were mixed with 4X Lilly buffer and denatured at 95°C for 5 min before subjecting to SDS gel electrophoresis.

2.2.5.2 Immunoblotting

$5x10^6$ wild-type pre-B cells after RhoA Inhibition or *RhoA*$^{fl/fl}$ Cre ER^{T2} or ER^{T2} pre-B cells after RhoA deletion were used for immunoblotting. Cells were harvested and lysed with RIPA buffer containing protease and phosphatase cocktail. Lysates were cleared by centrifugation at 13200 rpm for 15 min at 4°C. The supernatant, which has proteins, was collected and transferred to a fresh vial. Protein concentration was determined using Bradford reagent as described above. Equal amounts of denatured protein were separated on a 10% gel by SDS-electrophoresis and transferred onto a PVDF membrane. The membrane was incubated for 1 hr with blocking buffer (TBS/0.1% Tween-20; Sigma) with 5% BSA (Serva) and incubated overnight with the primary antibody diluted in blocking solution. Antibodies used for immunoblotting are listed in materials

After the washing steps, the membrane was incubated for 1 hr at room temperature (RT) with horseradish peroxidase (HRP)-coupled secondary antibodies diluted in blocking buffer. Membranes were washed off to remove excess antibodies and stained proteins were detected with an ECL Ultra Solution kit (Lumigen) on a Fusion SL advanced imaging system (Vilber Lourmat).

2.2.5.3 Stripping of western blots

Blots were developed sequentially with several antibodies. For this, membranes were incubated with Stripping buffer (Thermo Fisher Scientific) under shaking conditions for 15 mins. The Stripped membranes were washed and incubated with blocking buffer for 1 hour at room temperature. Subsequently, the blot was incubated with primary and secondary antibodies as explained above.

2.2.5.4 Proximity Ligation Assay (PLA)

$2x10^5$ cells were placed onto eight-well diagnostic microscope slides and left for 30 min at 4°C. The cells were then fixed with 4% paraformaldehyde at room temperature for 15 min. The slides were washed in PBS, and the cells were fixed at 37° for 20 min. PLA was performed on fixed and permeabilized cells following the manufacturer's instructions. In brief, the primary Antibodies (Abs) were detected with secondary anti-rabbit or anti-goat IgG Abs conjugated with PLA-PLUS and PLA-MINUS probes. The PLA probes were incubated with a ligation solution containing oligonucleotides and ligase. In the case of close proximity between the PLA probes, the oligonucleotides hybridized with the probes, forming a circle, which was then amplified in the following step by a polymerase diluted in the polymerization solution Fluorophore-labelled detection probes hybridized to the rolling circle amplification products, resulting in quantifiable signals. The cells were visualized using a fluorescence microscope (Leica) and quantitative analysis was performed with ImageJ and BlobFinder software.

2.2.5.5 Enzyme-Linked Immunosorbent Assay (ELISA)

For the anti-IgM and anti-IgG ELISAs, 96-well plates (NUNC, maxisorp) were coated with polyclonal anti-mouse IgM or IgG antibody (SouthernBiotech) and blocked with buffer containing 1% BSA. Dilutions of anti-mouse IgM/IgG (SouthernBiotech) were used as standard. The concentration of IgM and IgG in the supernatants was determined by detection with alkaline-phosphatase-labelled anti-mouse IgM or IgG (Southern Biotech), respectively. P-nitrophenylphosphate (PNPP, Genaxxon) in diethylamine buffer was added and data were acquired at 405 nm using a Multiskan FC ELISA plate reader (Thermo Scientific).

2.2.6 Molecular biology and biochemical methods

2.2.6.1 Competent cell preparation

XL10-Gold or DH5α E. coli strains were cultured in LB agar plate without antibiotics overnight at 37°C. The next day one colony from desired strain was picked and inoculated into a 3 ml SOC medium followed by 8-9 hours of incubation at 37°C with shaking. Later to 250 ml SOC medium, 1:2000 diluted pre-culture was added and incubated overnight at 25°C. Once the O.D of the culture reach a value between 0.4- 0.6, the culture was placed on ice for 10 mins. The culture was spun down at 2500 rpm for 10 min at 4°C and the pellet was resuspended in 80 ml of pre-cooled TB buffer, followed by a 10 min each incubation on ice and centrifugation at 2500 rpm. The pellet was resuspended in 20 ml pre cooled TB buffer and DMSO was added at 7% final concentration followed by 10 min incubation again on the ice. Aliquots of 100 µl competent bacteria were transferred into autoclaved pre-cooled Eppendorf tubes and stored at -80°C.

2.2.6.2 Transformation

Competent cells were thawed on ice for 20 min. Afterwards, 10 ng plasmid DNA or 10 µl of ligation product were mixed with 30-50 µl of competent cells and incubated on ice for 10 min. Competent cells were permeabilized by heat shock at 42°C for 45 sec and followed by 5 min incubation on ice. Transformed cells were plated on to LB plate containing 100 µg/ml of ampicillin and incubated overnight at 37°C.

2.2.6.3 Plasmid DNA extraction

Based on the amount of plasmid DNA needed, bacteria were grown in 5 ml or 50 ml of LB media supplemented with 100 µg/ml ampicillin and incubated at 37°C with shaking at 250 rpm overnight. Plasmid DNA was isolated using a QIAGEN plasmid Mini or Maxi prep kit according to the manufacturer´s protocols. In short, bacterial cells were subjected to alkaline lysis followed by adsorption of the DNA onto a silica membrane in the presence of high salt. The DNA was eluted in nuclease-free water or TE buffer. DNA concentration and purity were determined using a Nano spectrophotometer.

2.2.6.4 Genomic DNA isolation

For genomic DNA isolation, cells were spun down for 5 min at 1200 rpm at 4 °C. The supernatant was aspirated and the cells were lysed by resuspending the pellet in 500 µl of Proteinase K containing lysis buffer. The sample was incubated at 56°C for 1 hour under

continuous shaking (650 rpm). Subsequently, 500 µl isopropanol was added to the digest, mixed by vortexing, followed by centrifugation at 13000 rpm at 4°C for 10 min. Afterwards, the supernatant was discarded, the pellet was washed with 70% ethanol at 13000 rpm at 4°C for 10 min. Ethanol was poured off, samples were incubated at 37°C with the open lid so that ethanol is completely evaporated. Later DNA was resuspended in 100 µl TE buffer and stored at -20°C for further use. DNA concentration and purity were determined using a Nano spectrophotometer.

2.2.6.5 Polymerase chain reaction (PCR)

To amplify specific DNA sequences Polymerase chain reaction (PCR) is used. In a PCR reaction target DNA sequence (template) is amplified by high-fidelity thermostable DNA polymerase upon the binding of specific oligonucleotides (primers) complementary to the 5' and 3' sequence of template DNA. The temperature of different steps depends on the polymerase used and the melting temperature (Tm) of the primers.

Table 5: Standard PCR cycling protocol

	Steps	Temperature	Time
1	Initial denaturation	94°C	3 min
2	Denaturation	94°C	30 sec
3	Annealing temperature	Depending on primers	30 sec
4	Elongation	72°C	1 min/kb
5	Final elongation	72°C	3 min
6	Store	12°C	

2.2.6.6 Agarose gel electrophoresis

With this method, DNA fragments are separated according to their size. 1% agarose gels were prepared by melting 1.5 g agarose in 150 ml 1x TAE buffer. When the agarose was completely melted, ethidium bromide was added to a final concentration of 0.25 µg/ml. The solution was filled into a gel chamber and a comb was added. After polymerization of the gel, the samples were mixed with 5x DNA loading dye and loaded on the gel together with a standard size marker. The gel was run at 100-130 V in 1x TAE buffer. The detection of the DNA bands was carried out under UV light of 312-360 nm. The size of the bands was determined by comparison to the defined marker bands.

2.2.6.7 RNA extraction

Total RNA was isolated using ReliaPrep™ RNA Cell Miniprep System (Promega), and PicopureTM RNA isolation kit (Arcturus) according to the manufacturer's instruction. The quality of the isolated RNA was either determined by running 0.3 µg RNA on 1% Agarose gel electrophoresis or by a Nanophotometer. A ratio of absorbance at 260 and 280 nm gives the quality of RNA isolated. Pure RNA will have a ratio of ~ 2. Anything above or below this value gives a hint of contamination. RNA concentration was determined using Nanophotometer by measuring the optical density at a wavelength of 260 nm.

2.2.6.8 cDNA synthesis

cDNA was synthesized using a High-capacity RNA to cDNA kit (Applied Biosystems) and RevertAid Reverse Transcriptase kit (Thermo Fischer).

Table 6: Master mix for cDNA synthesis using RevertAid Reverse transcriptase Kit:

Components	Volume of reaction	
	+ RT reaction	-RT control
Reverse transcriptase buffer (5X)	4 µl	4 µl
Mgcl2 (25mM)	2 µl	2 µl
Oligo (dT)18 primer	1 µl	1 µl
DTT (0.1 M)	0.5 µl	0.5 µl
dNTPs (25 mM)	0.4 µl	0.4 µl
Reverse transcriptase (RT)	0.4 µl	-
RNase free H_2O	1,6 µl	2 µl
Total per reaction	10 µl	10 µl

cDNA synthesis master mix was added to 10 µl of RNA, to obtain a final volume of 20 µl, and incubated at 42°C for 90 min. cDNA synthesised without the addition of reverse transcriptase was used as a negative control to ensure that there is no gDNA contamination.

Table 7: Master mix for cDNA synthesis using High-capacity RNA to cDNA kit:

Components	Volume of reaction	
	+ RT reaction	-RT control
2X RT buffer mix	10 µl	10 µl
20X RT enzyme mix	1 µl	-
RNA sample	Up to 9 µl	Up to 9 µl
Nuclease free H_2O	Up to 20 µl	Up to 20 µl
Total per reaction	20 µl	20 µl

cDNA synthesis master mix was aliquoted and spun down to eliminate air bubbles. The reaction was incubated at 37°C for 60 min and the reaction was stopped by heating at 95°C for 5 min. Synthesised cDNA can be used for immediately used real-time PCR or stored at -20°C for further use.

RhoA deletion was done as described before. For analysing the presence of floxed and deleted RhoA allele, RhoA deletion PCRs were performed on cDNA using primers listed in the material section. Dream Taq DNA polymerase was used for the PCR reactions and done as mentioned above. Actin was used as the loading control.

2.2.6.9 Quantitative real time PCR (qRT-PCR) using Taq Man probes

Quantitative real-time PCR (qRT-PCR) analyses were performed using TaqMan PCR probe mixes with TaqMan gene expression master mix as given below (Applied Biosystems). qRT-

PCR data were acquired on a StepOnePlus Real-Time thermocycler (Applied Biosystems) and analyzed with the StepOne Software version 2.3. Relative quantification (RQ) was calculated using the $2^{-\Delta\Delta C_T}$ equation.

Table 8: Master mix for qRT-PCR using Taq-Man probes

Components	1X
TaqMan gene expression master mix	10 µl
TaqMan probe mix	1 µl
Nuclease free H_2O	7 µl
Template (cDNA)	2 µl
Total volume	20 µl

Quantitative real time PCR (qRT-PCR) using SYBR-GREEN probes

Quantitative real-time PCR (qRT-PCR) analyses were performed using SYBR-GREEN probe mixes with SYBR-GREEN master mix as given below (Applied Biosystems). qRT-PCR data were acquired on a StepOnePlus Real-Time thermocycler (Applied Biosystems) and analyzed with the StepOne Software version 2.3. Relative quantification (RQ) was calculated using the $2^{-\Delta\Delta C_T}$ equation.

Table 9: Master mix for qRT-PCR using SYBR-GREEN probes

Components	1X
SYBR-Green master mix	2 µl
Forward primer (10 pM)	5 µl
Reverse primer (10 pM)	5 µl
Nuclease free H_2O	5.5 µl
Template (cDNA)	2.5 µl
Total volume	20 µl

2.2.7 Statistical Analysis

Graphs were generated and statistical analysis was performed using GraphPad Prism (Version 6.0f) software. The number of experimental replicates or mice is stated in the figure description. Assumption of normality was tested by the statistical method D'Agostino and Pearson test. Based on whether the data follow a Gaussian normal distribution or not, either parametric or non-parametric tests were performed respectively. When the sample size is too small to perform a normality test, non-parametric tests were chosen. Tests used are one-sample t-test, unpaired t-test with Welch's correction, Mann-Whitney U test, and one-way ANOVA (Kruskal Wallis test) with multiple comparisons test. P-values were mentioned in the respective figure legends. P-values <0.05 were considered to be statistically significant (n. s. = not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

3 Results

3.1 GEF-H1 is a novel interaction partner of SLP-65

Previous work in our group using split ubiquitin yeast two-hybrid assay have identified 10 potential interaction partners of SLP-65 together with the known SLP-65 binders. One of the novel candidates identified was Rho Guanine nucleotide exchange factor 2 (*Arhgef2* also known as *Gef-h1*) (Vadakumchery et al., 2022). GEF-HI is a guanine nucleotide exchange factor known to be regulating the activity of the Rho family of GTPases (Van Aelst and D'Souza-Schorey, 1997) and small GTPases are shown to be essential for B cell development (Zhang et al., 2012). This led us to the hypothesis that GEF-H1 is important for SLP-65-mediated signalling in B cells. To further validate whether SLP-65 interacts with GEF-H1 at the cellular level, we performed an immunoprecipitation in DG75EB/HA-RhoA, a human Burkitt lymphoma cell line modified to delete RHOA. For this DG75EB/HA-RhoA cells were stimulated with IgM F(ab)'2 antibody for different time points to stimulate SLP-65 and later IP was performed using anti-SLP-65 antibody. We observed an interaction of SLP-65 with GEF-H1 at all time points and also before stimulation (Figure 9A). Together with this, we also found the interaction of SLP-65 with PLCγ2, a well-known interaction partner of SLP-65 (Figure 9A). Taken together these findings indicate that SLP-65 interacts with GEF-H1 at the endogenous protein level. To confirm that IgM stimulation worked in DG75EB/HA-RhoA cell line, we performed an immunoblot analysis. As described above DG75EB/HA-RhoA cells were stimulated with IgM F(ab)'2 antibody for different time points to stimulate SLP-65 and immunoblot was performed on total cell lysate (TCL). Phosphorylated SLP-65 (p-SLP-65), the activated form of SLP-65 showed elevated expression in IgM stimulated cells compared to unstimulated control. We also observed the expression of GEF-H1 and SLP-65 in unstimulated control (Figure 9B).

To further investigate the interaction of SLP-65 and GEF-H1 in B cells, we used a Proximity ligation assay (PLA) (Becker et al., 2017; Söderberg et al., 2008). The working principle of PLA is that primary antibodies from two different species are used to target two different proteins in the cell. Corresponding secondary antibodies are labelled with DNA probes. When two proteins are in close proximity, then DNA probes will hybridize and undergo rolling circle amplification. The amplified DNA will be detected as a bright red fluorescence using a high-resolution microscope. A significant increase in red fluorescent dot indicating an increased interaction between SLP-65 and GEF-H1 was observed compared to control (Figures 9C). The control used was only secondary antibody against GEF-H1 along with SLP-65 primary antibody. The result suggests that SLP-65 and GEF-H1 are localized at close proximity of 10-40 nm at the cellular level in B cells. Using these different approaches, we have confirmed that GEF-H1 is a novel interaction partner of SLP-65.

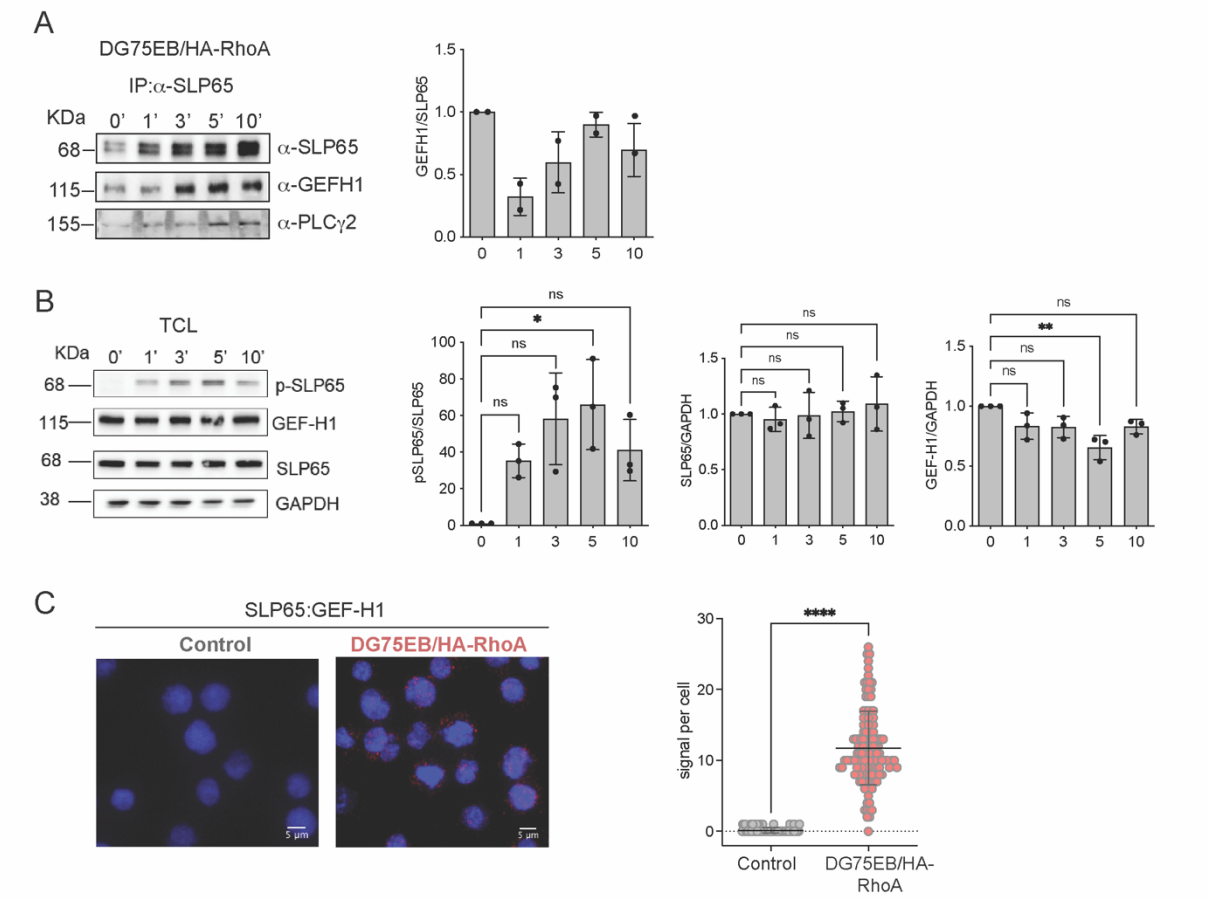

Figure 9: GEF-H1 is a novel interaction partner of SLP-65: (A*) Left- immunoprecipitation (IP) followed by western blot analysis for the interaction of endogenous SLP-65 with GEF-H1 in DG75EB/HA-RhoA cell line upon BCR stimulation. Cells were stimulated with anti-IgM F(ab)′2 antibody for the indicated time and IP was carried out with anti-SLP-65 antibody. PLCγ2, a known interactor of SLP-65 is used as a positive control. Results are representative of 2 independent experiments. Right- quantification of GEF-H1 with respect to SLP-65 after IP. (B) Left- DG75EB/HA-RhoA were stimulated with anti-IgM F(ab)′2 antibody for the indicated time and the expression of p-SLP-65, SLP-65, and GEF-H1 in the total cell lysate (TCL) were analysed by western blot. Right- quantification of western blot. pSLP-65 is normalized to SLP-65 whereas SLP-65 and GEF-H1 are normalized to GAPDH. Results are representative of 3 independent experiments (C*) Proximity ligation assay (PLA) showing the association of SLP-65 and GEF-H1 in DG75EB/HA-RhoA cell line. Secondary antibodies alone were used in the control. Right- quantification of the number of signals per cell, error bars represent mean ± SD. Unpaired t-test with Welch's correction. PLA experiments were done in association with research group of Dr. Elias Hobeika, Institute of Immunology. (* are modified from Vadakumchery et al., 2022, Main Figure 1A and B, page 4 of 19, © by the authors).

3.2 SLP-65 interacts with RHOA and regulates its activation

Rho GTPases activity is regulated by GEF that enhances the exchange of bound GDP to GTP (Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000; Bos et al., 2007). GEF-H1, which we identified as a novel interaction partner of SLP-65 is the known activator of RHOA, RHOB and RHOC (Bos et al., 2007). This led us to investigate whether SLP-65 also interacts with RHOA, a member of the Rho family of GTPases that is crucial for B lymphopoiesis (Zhang et al., 2012). To test the interaction of SLP-65 with RHOA we employed PLA. We observed a

significant increase in red fluorescence signals indicating an increased interaction between SLP-65 and RHOA (Figure 10A). To investigate whether stimulation of BCR and subsequent SLP-65 activation further enhances the association of SLP-65 and RHOA, we stimulated DG75EB/HA-RhoA cells with IgM F(ab)'2 antibody for different time points and the interaction was analysed using PLA. We found that phosphorylated SLP-65 interacts with RHOA as early as 3 minutes (Figure 10B). Next, we ask the question that whether inducing activity of SLP-65 results in RHOA activation. To address this, we used a triple knock-out (TKO) cell system reconstituted with inducible SLP-65. TKO cells are early pre-B cells derived from RAG2, λ5 and SLP-65 triple knock-out mice reconstituted with the inducible form of SLP-65 (ER^{T2}-SLP-65) and pre-BCR (μHC and λ5). When stimulated with 4-OHT, SLP-65 is activated and leads to calcium mobilization (Meixlsperger et al., 2007; Storch et al., 2007). We found that 4-OHT mediated activation of SLP-65 in TKO cells led to the significant increase in RHOA activity after 5 min and even up to 30 min (Figure 10C). Thus, our findings show that the interaction of SLP-65 to RHOA leads to its activation.

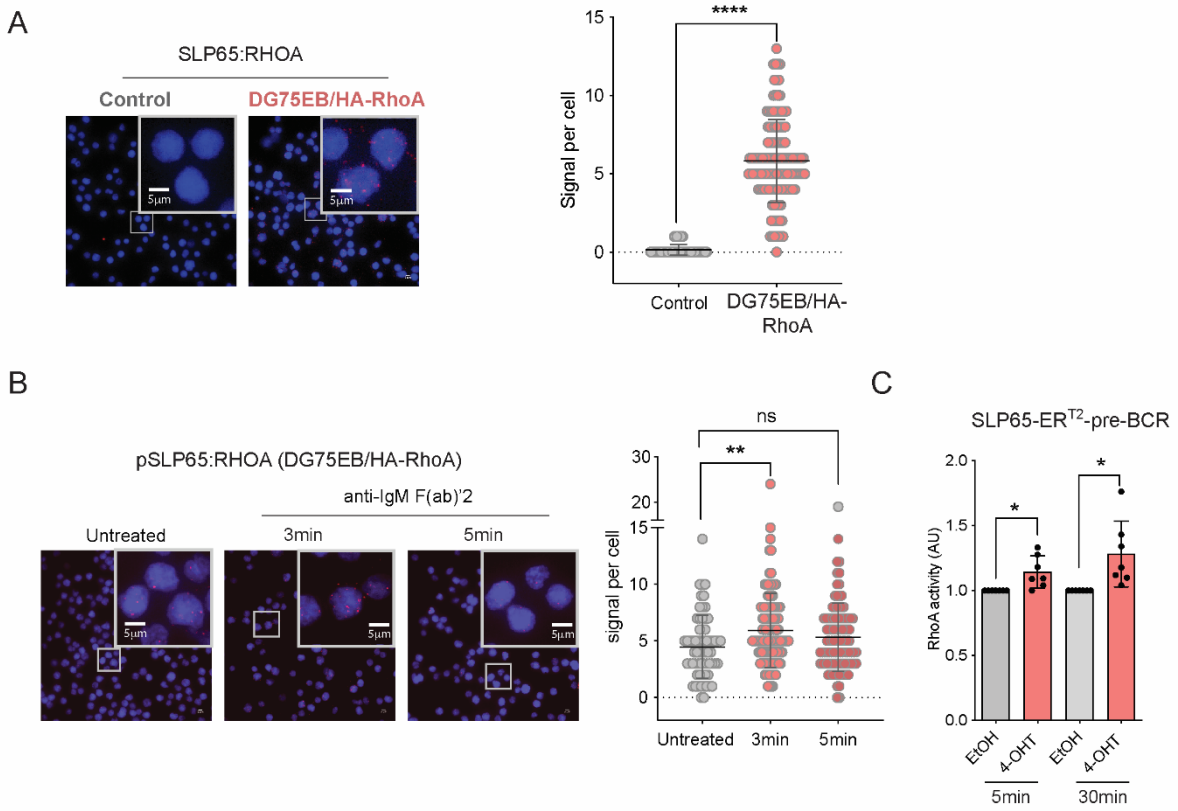

Figure 10: SLP-65 interacts with RHOA and regulates its activation: (A*) Left- PLA showing the association of RHOA with SLP-65 in DG75EB/HA-RhoA cell line. Secondary antibodies alone were used in the control. Right- quantification of the number of signals per cell, bars represent mean ± SD. An unpaired T-test with Welch's correction was used for statistical significance. (B*) Left- DG75EB/HA-RhoA were stimulated with anti-IgM F(ab)′2 antibody for the indicated time and PLA was performed to show the association of RHOA with phosphorylated SLP-65. Untreated cells were used as a control. Right- quantification of the number of signals per cell, error bars represent mean ± SD. Statistical significance was calculated using the unpaired T-test with Welch's correction. (C*) Detection of total cellular RHOA activity by G-LISA RHOA activation assay in TKO cells derived from RAG2, λ5 and SLP-65 triple knock-out mice, which were reconstituted with inducible form of SLP-65 (ER^{T2}-SLP-65) and pre-BCR (μHC and λ5). After reconstitution, the cells were starved for 72 hours and induced with 2 μM 4-OHT or the same volume of solvent EtOH for 5 and 30 min and

lysed. Equal amounts of cell lysates were used and the total cellular RhoA activity was determined by the G-LISA RhoA activation assay. Statistical significance was calculated using one sample t-test. Data shown are from 7 independent experiments. (* are modified from Vadakumchery et al., 2022, Main Figure 2A, B and C, page 5 of 19, © by the authors).

3.3 RHOA connects SLP-65 to PI3K-PTEN signalling

RHOA is previously shown to regulate the localization and activity of PTEN and thereby counteracting the PI3K signalling (Li et al., 2005). SLP-65 is an adaptor protein that down-regulates PI3K activity, thereby inducing differentiation of pre-B cells (Herzog et al., 2008). But the interaction partner/s that connect(s) SLP-65 to PI3K signalling was missing. This led us to hypothesize that GEF-H1-RHOA may provide the molecular platform that connects SLP-65 to PI3K signalling. To investigate this, we examined the effect of RHOA inhibition on PI3K signalling. To inhibit RHOA, we treated wild-type bone marrow-derived pre–B cells with 1 and 5 µg/ ml of cell-permeable Rho-specific inhibitor, CT04 for 16 hours (CT04 inhibits Rho GTPases exclusively RHOA, RHOB and RHOC by ADP-ribosylation on effector binding domain of GTPase) or left untreated as a control. The inhibition of RHOA in pre-B cells leads to a significant increase in AKT activation shown by the phosphorylation of serine 473 (Figure 11) and a slight but significant upregulation in PTEN expression (Figure 11). We observed no significant change in RHOA expression (Figure 11). Since RHOA has been shown to regulate the localization of PTEN, using TKO cells PTEN localization upon SLP-65 induction was analysed. PTEN activation and membrane localization was observed after SLP-65 induction. To further confirm that SLP-65-induced PTEN localization was mediated through RHOA, cells were treated with RHOA inhibitor, which blocked PTEN clustering and following recruitment to the membrane (Vadakumchery et al., 2022). Taken together these data suggest that SLP-65 mediated activation of RHOA leads to PTEN activation and this physically links SLP-65 to the PI3K signalling pathway.

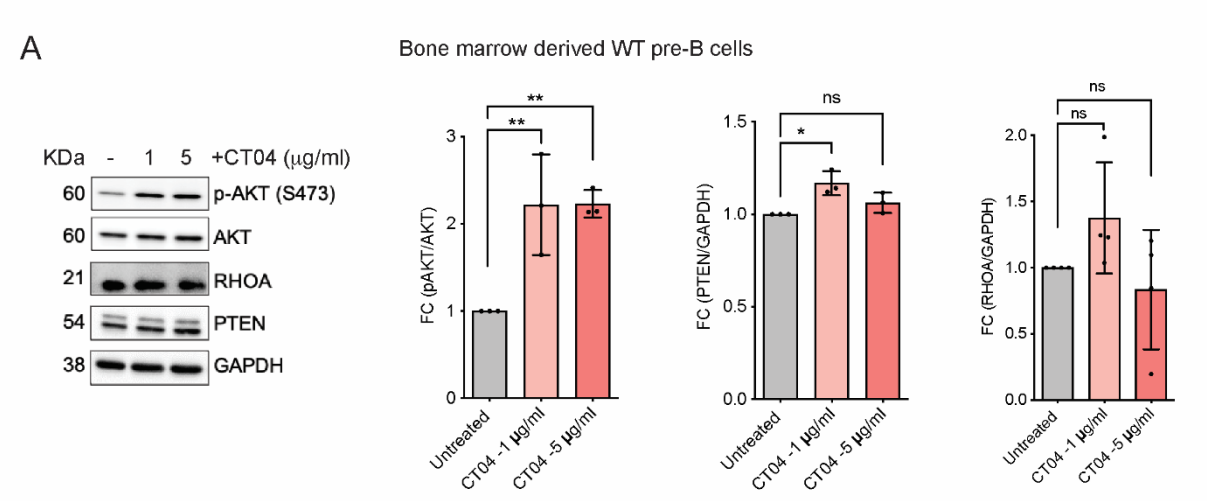

Figure 11*: SLP-65 regulates PI3K-PTEN signalling through RHOA: Bone marrow cells from wild-type (WT) mice were cultured for 5 days in a medium with IL-7 and treated with the indicated concentrations of the Rho-specific inhibitor CT04 or left untreated for 16 hrs. Total cell lysates were prepared and p-AKT, AKT, PTEN, and RHOA, expression was analyzed using western blot. GAPDH served as a loading control. Right is the quantification of western blot- PTEN and RHOA expression are normalized to GAPDH whereas p-AKT is normalized to total AKT. Statistical significance was calculated using ordinary one-way ANOVA with multiple comparisons. Data are representative of at least 3 independent experiments. (* is modified from Vadakumchery et al., 2022, Main Figure 3A, page 6 of 19, © by the authors)

3.4 RHOA is essential for early B cell development

3.4.1 B cell specific *RhoA* deletion leads to developmental block at pro B cell stage.

Our results show that RHOA connects SLP-65 activation to PI3K-PTEN signalling. SLP-65 is a crucial factor that controls the pre-B cell differentiation mainly by regulating the PI3K-AKT-FoxO pathway and *SLP-65* deficient mice show partial block in B-cell development specifically at pre-B cell stage (Herzog et al., 2008; Jumaa et al., 1999). Previously our group showed that PI3K signalling regulates pre-B cell differentiation and the regulation of PI3K activity is required for early B cell development (Abdelrasoul et al., 2018; Setz et al., 2018). Furthermore, it has been shown that inactivation of *RhoA* in hematopoietic stem cells using Mx-cre leads to an increase in common lymphoid progenitor (CLP) together with a reduction of Pro/Pre/Immature B cell compartments in bone marrow (Zhang et al., 2012). This underlines the significance of RhoA in B lymphopoiesis. To assess the importance of RHOA in early B cell development, homozygous mice carrying a floxed *RhoA* allele ($RhoA^{fl/fl}$ in which exon 3 of the *RhoA* gene is flanked by two lox P sites) were crossed with mice expressing Cre under the control of B cell-specific mb1 promoter (Hobeika et al., 2006). The early expression of mb1 in pro B cell enables efficient deletion of *RhoA* at an early stage before VDJ recombination. We analysed bone marrow cells from $RhoA^{fl/fl}$ $mb1^{+/hCre}$ by flow cytometry and found that B cell development is blocked at the pro B cell stage (Figure 12A and B). Further detailed analysis using different stage-specific cell surface markers revealed that *RhoA* deficient mice had a significant reduction in B cell population ($CD19^{+}B220^{+}$) in the bone marrow. The Pro B cell ($B220^{+}CD43^{+}$) compartment was not increased in *RhoA* deleted mice. In addition to a

significant decrease in pre-B cells ($B220^+CD25^+$) *RhoA* deficient mice also lacked immature ($B220^+IgM^+$) B cells.

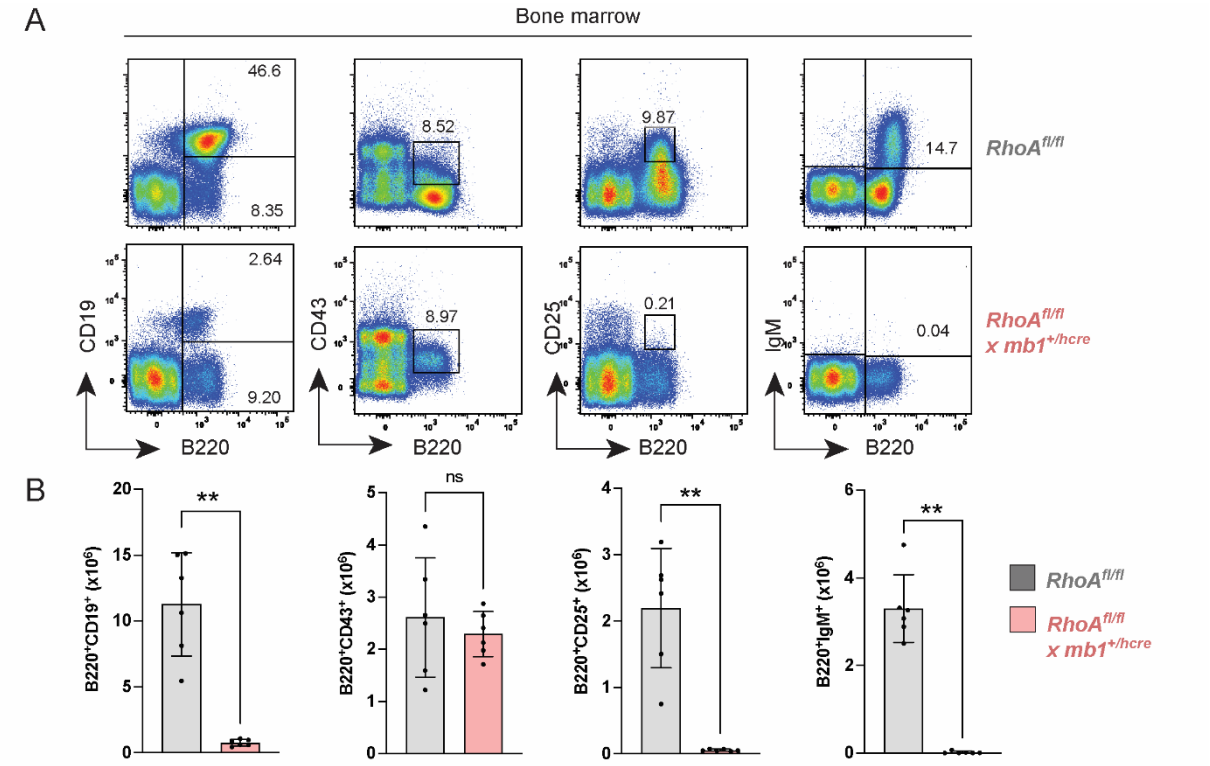

Figure 12: Complete block of early B cell development in *RhoA* deficient mice: (A*) Freshly isolated cells from the bone marrow of $RhoA^{fl/fl}$ and $RhoA^{fl/fl}$ $mb1^{+/hcre}$ mice were analyzed by flow cytometry for surface expression of the indicated markers. Representative data are from 6 individual mice. (B*) Absolute cell numbers of $B220^+CD19^+$, $B220^+CD43^+$, $B220^+CD25^+$and $B220^+IgM^+$ cells in the bone marrow from $RhoA^{fl/fl}$ $mb1^{+/hcre}$ mice (n=6) were compared with control $RhoA^{fl/fl}$ mice (n=6). Statistical significance was calculated using Mann-Whitney U test. Data shown are from 6 individual mice from each group. (* are modified from Vadakumchery et al., 2022, Main Figure 4A, page 7 of 19, © by the authors).

Flow cytometric analysis of the spleen revealed that $RhoA^{fl/fl}$ $mb1^{+/hCre}$ mice lack mature B cells ($CD19^+IgM^+IgD^+$) together with significant decrease in follicular and marginal zone B cells in the periphery (Figure 13A and B). The B cell developmental block observed in *RhoA* deficient mice was similar to the phenotype observed by inactivating the gene encoding *Pten* or *FoxO1* using mb1-cre (Alkhatib et al., 2012). Inactivation of both these genes at the pro-B cell stage resulted in the severe developmental block at the pro-B cell stage. Together these data show that RhoA is indispensable for early B cell development, especially for the pre-B cell differentiation.

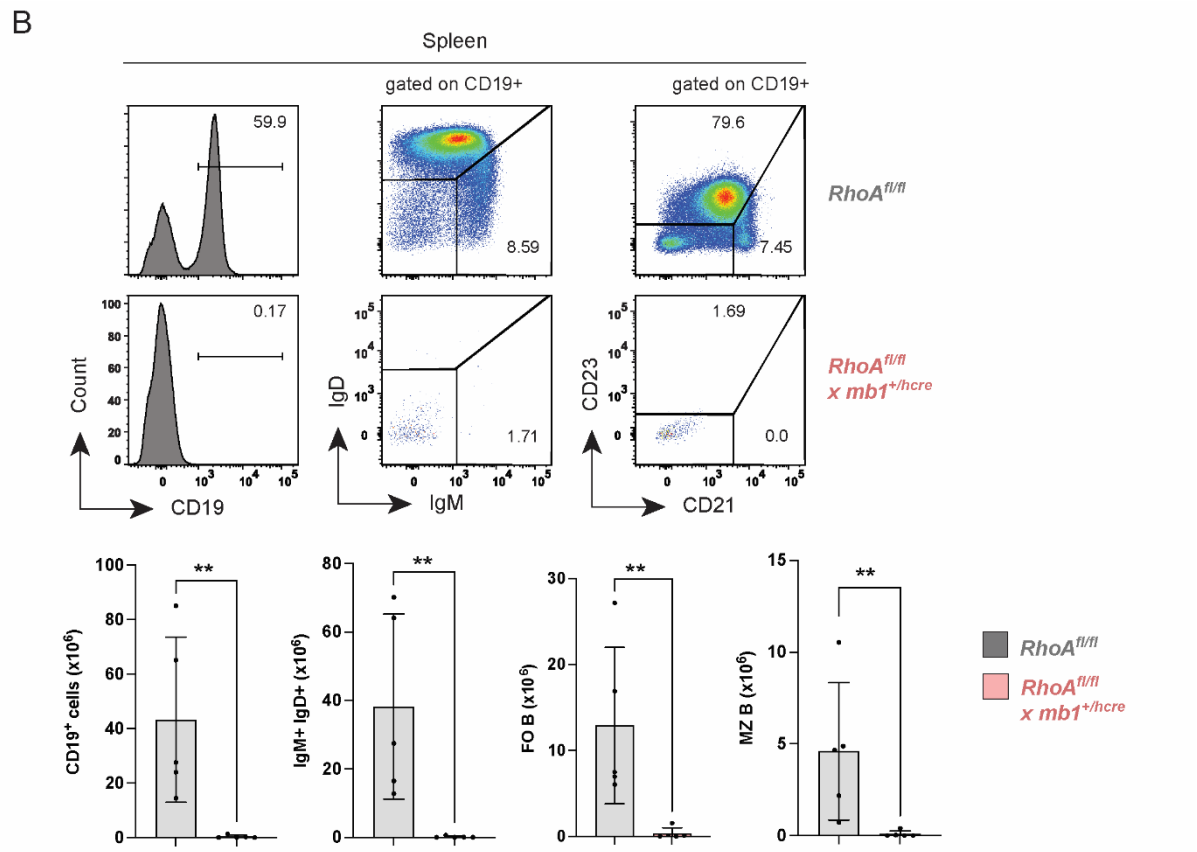

Figure 13: *RhoA* deficient mice lack mature B cells in the periphery: (A*) Splenic cells were isolated from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice and were analyzed by flow cytometry for surface expression of the indicated markers. Representative data are from 5 individual mice per group. (B*) Absolute cell numbers of CD19^{+}, IgM^{+} IgD^{+}, FO B and MZ. B cells in the spleen of *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice (n=5) were compared with control *RhoA*$^{fl/fl}$ mice (n=5). Statistical significance was calculated using Mann-Whitney U test. (* are modified from Vadakumchery et al., 2022, Main Figure 4B, page 7 of 19, © by the authors).

3.4.2 B cells precursors require RHOA for their enrichment in IL-7 dependent cultures

We have seen that *RhoA* deficient mice have impaired B cell development. Next, we wanted to further investigate the significance of RHOA in B cell survival and proliferation *in vitro*. For this, we isolated the bone marrow cells from *RhoA* deficient and control mice and monitored their growth in IL-7 dependent medium by flow cytometry. The proliferation of early B cells in bone marrow stromal cultures seems to be dependent on IL-7 (Namen et al., 1988). Unfortunately, we were not able to establish the IL-7 dependent in vitro cultures from RHOA deficient bone marrow precursor cells and B cells (CD19^{+}CD11b^{-}) were lost from the culture over a period of two weeks (Figures 14A and B). Previous work from our group has shown that *FoxO1* deficient precursor B cells from *FoxO1*$^{fl/fl}$ *mb1-cre* mice were unable to grow in IL-7-dependent in vitro culture (Alkhatib et al., 2012). Taken together, this data suggests that precursor B cells require RHOA for their survival in *in vitro* culture.

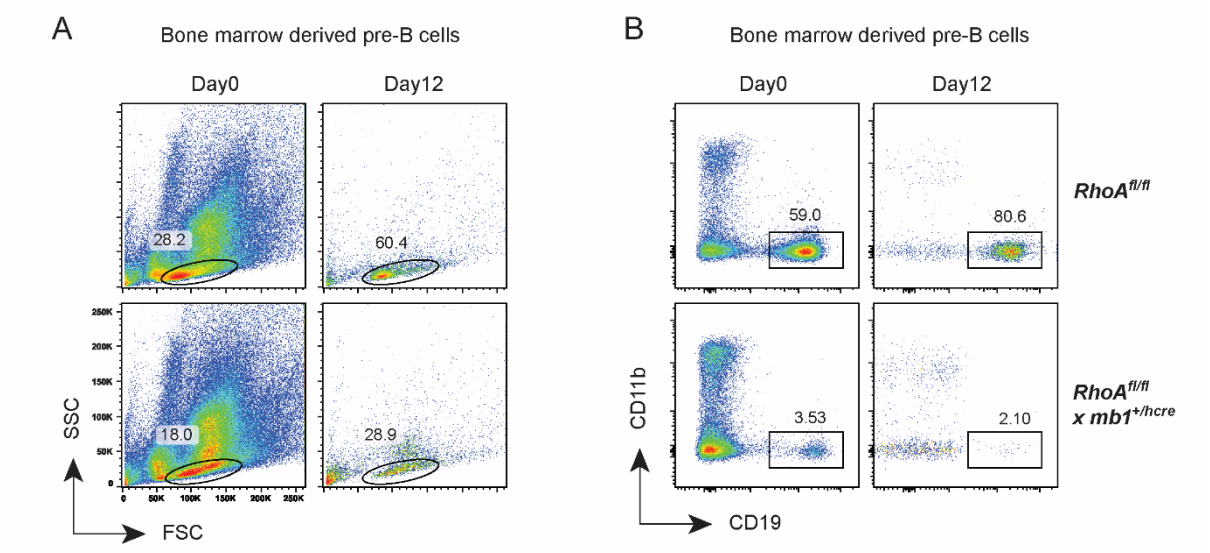

Figure 14: *RhoA* deficient pre-B cells are lost when cultured in IL-7 containing medium: (A) Bone marrow cells were isolated from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice and cultured for 12 days in a medium supplemented with IL-7. Representative flow cytometric analysis for the forward and side scatter is shown for each genotype at the indicated time point. (B) CD19 and CD11b surface expression were analysed by flow cytometry at the indicated time points. Data are representative of at least 2 mice per genotype.

3.4.3 RHOA is essential for the survival and differentiation of pre-B cells

Our data shows that RHOA is indispensable for the development of B cells in vivo. Since we could not establish an IL-7-dependent *RhoA* deficient bone marrow B cell culture, to investigate the role of RHOA in vitro we used an inducible *RhoA* deletion system. For this, we retrovirally transduced bone marrow-derived pre-B cells from *RhoA*$^{fl/fl}$ mice with 4-OHT inducible Cre (Cre ER^{T2}) or its control ER^{T2}. Cells were treated either with 4-OHT or its control ethanol for 3 days to induce *RhoA* deletion and the viability of the treated cells was analysed by flow cytometry. We found that *RhoA* deleted pre-B cells were significantly lost from culture 3 days after tamoxifen induction compared to the controls (Figure 15A). We further confirmed *RhoA* deletion using a PCR at the mRNA level (Figure 15B). We found that *RhoA* expression was absent in RHOA deletion-induced cells compared to controls. Together these data show that RHOA is essential for the survival of pre-B cells.

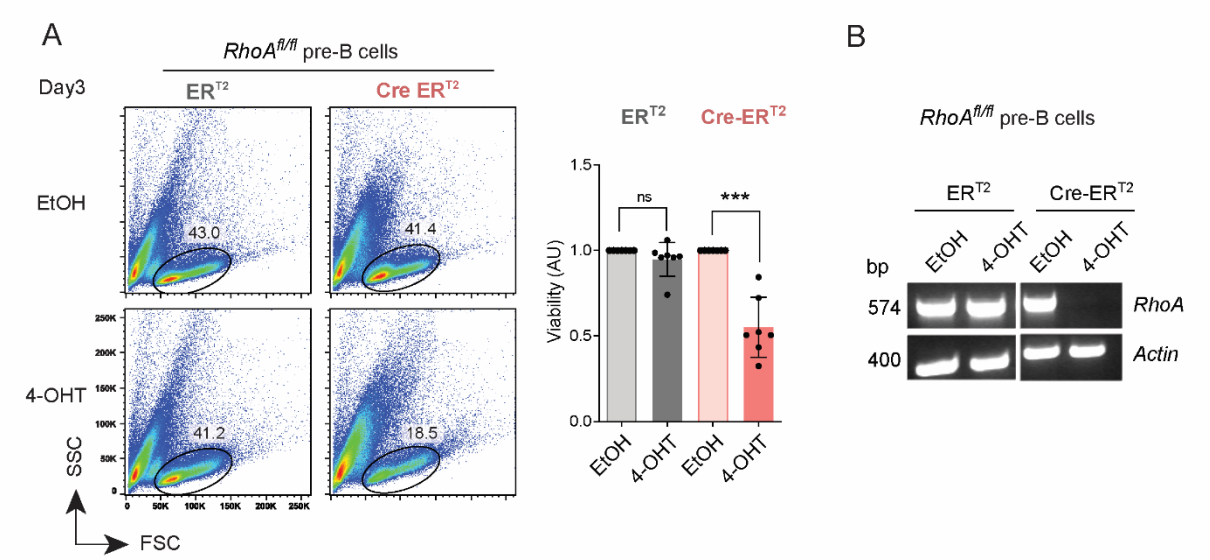

Figure 15: *RhoA* is essential for the survival of pre-B cells: (A*) Left- Bone marrow-derived cells from *RhoA*$^{fl/fl}$ mice were retrovirally transduced with Cre-ER^{T2} or its control ER^{T2} construct. Cells were treated with 2 µM 4-OHT or the solvent ethanol (EtOH) to induce Cre recombination. The viability of the cells was measured 3 days after 4-OHT treatment by flow cytometry. Right- Quantification of viable cells after 4-OHT treatment in comparison with the controls. Statistical significance was calculated using one-sample t-test. Data are representative of 7 individual experiments. (B*) 48 hours after Cre induction, RNA was isolated and cDNA was synthesised. *RhoA* expression was analysed at the mRNA level using semi-quantitative RT-PCR. Actin was used as the loading control. Representative figure from 3 independent experiments. (* are modified from Vadakumchery et al., 2022, Main Figure 5A, page 8 of 19, Supplementary figure S3A, and B, page 3 of 11 © by the authors).

Our results suggest that SLP-65 mediated RHOA activation regulates PTEN expression and interferes with PI3K signalling. Since *RhoA* deficient mice have a developmental block at the pro B cell stage, we used our *RhoA* inducible deletion system (as explained above) to identify the functional association between RHOA and PTEN-PI3K signalling. Inducible deletion of *RhoA* led to a small but significant reduction in PTEN expression (Figure 16). We also observed small reduction in total FoxO1 together with increased relative phosphorylation of FoxO1 at serine 256 indicating its reduced transcriptional activity and subsequent degradation (Greer and Brunet, 2005) in *RhoA* deleted cells compared to controls (Figure 16). Our findings indicate that RHOA is required for the proper PTEN expression and regulation of the PI3K signalling pathway in pre-B cells.

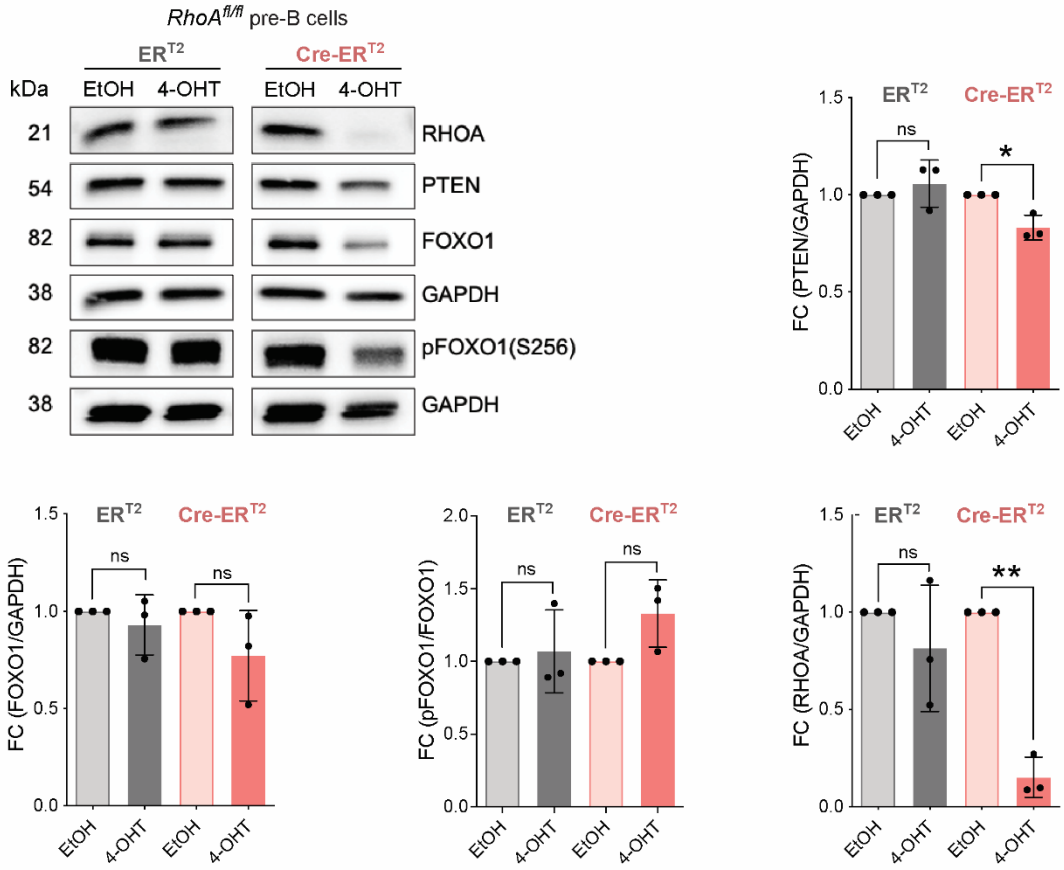

Figure 16*: *In vitro RhoA* deletion leads to reduced PTEN expression in pre-B cells: Left- Bone marrow-derived cells from *RhoA*$^{fl/fl}$ mice were retrovirally transduced with Cre-ER^{T2} or its control ER^{T2} construct. Cells were treated with 2 µM 4-OHT or the solvent EtOH to induce Cre recombination. Immunoblot analysis showing expression of RhoA, PTEN, p-FOXO1 and FOXO1 48 hours after 4-OHT or EtOH treatment. Right and below- quantification of western blot. PTEN, RHOA, and FOXO1 were normalized to GAPDH whereas p-FOXO1 is normalized to FOXO1. Fold change (FC) is represented. Statistical significance was calculated using one sample t-test. Data are representative of 3 individual experiments. (* is modified from Vadakumchery et al., 2022, Main Figure 5C, page 8 of 19, Supplementary figure S3C, page 3 of 11 © by the authors).

Previously our group and others have shown that the early B cell developmental block observed in *Pten* or *FoxO1* deleted mice was primarily due to defective *IgH* gene recombination (Alkhatib et al., 2012; Amin and Schlissel, 2008; Dengler et al., 2008). FoxO1 controls immunoglobulin (*Ig*) gene recombination mainly by regulating the expression of recombination activating genes *Rag 1* and *2* (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008). Moreover, FoxO1 is the downstream target of P13K signalling and its activity also depends on PTEN (Kops et al., 2002; Leslie and Downes, 2002; Nakamura et al., 2000; Setz et al., 2019). *RhoA* deficient mice showed a similar phenotype observed after inactivating *Pten* or *FoxO1* at pro B cell stage using mb1-cre. This led us to hypothesize that severe B cell developmental block observed in *RhoA* deficient mice at the pro B cell stage is also due to defective Ig recombination. To assess this, we measured the expression of *Rag1* and *Rag2* using q-RT PCR by inducibly deleting *RhoA*. We found that deletion of *RhoA* in pre-B cells resulted in a significant reduction in the expression level of *Rag 1* and *2* in comparison to the controls

(Figure 17). Reduced *Rag1* and *Rag2* expressions are in agreement with our previous observation that RHOA deleted cells show increased FOXO1 phosphorylation. Thus, our result shows that RHOA is indispensable for the proper induction of recombination activating genes and light chain gene recombination in pre-B cells.

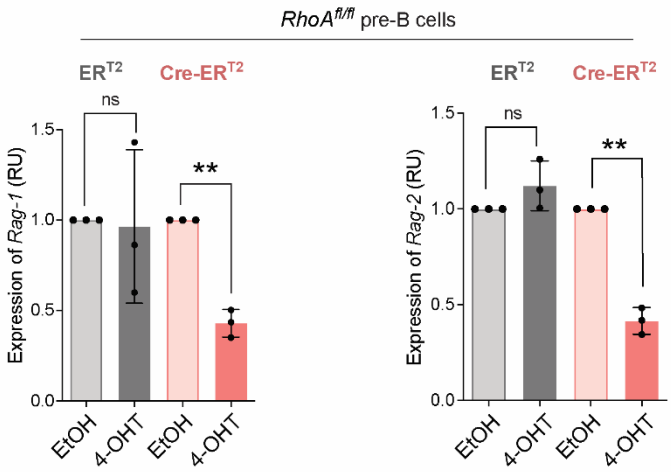

Figure 17*: Recombination activating genes are down regulated in *RhoA* deleted pre-B cells: Bone marrow-derived cells from *RhoA*$^{fl/fl}$ mice were retrovirally transduced with Cre-ER^{T2} or its control ER^{T2} construct. Cells were treated with 2 µM 4-OHT or the solvent EtOH to induce Cre recombination. *Rag-1* and *Rag-2* expression levels were measured using quantitative RT-PCR 48 hours after *RhoA* deletion. Results are shown as relative units normalized to levels of EtOH treated cells. Data are representative of 3 individual experiments. Statistical significance was calculated using one sample t-test (* is modified from Vadakumchery et al., 2022, Main Figure 5D, page 8 of 19, Supplementary figure S3D, page 3 of 11 © by the authors).

3.4.4 Ectopic expression of RHOA restores in vivo differentiation block

We have shown that RHOA is essential for B cell development, especially for pre-B cell differentiation. To further investigate whether reconstitution of RHOA can overcome the differentiation block found at the pro B cell stage in *RhoA* deficient B cells, we purified the CD19^{+} cells from the bone marrow of *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice using MACS sorting. Using flow cytometric analysis, we further confirmed that CD19 purified cells from *RhoA*$^{fl/fl}$ *mb1*$^{+/hCre}$ had no expression of IgM^{+}kappa^{+} cells on their surface (Figure 18A). Kappa surface expression is a marker for light chain recombination in pre-B cells (Herzog et al., 2008; Nemazee, 2017). Further, we transduced CD19 enriched bone marrow B cells with RHOA-IRES-GFP retroviral expression vector or its empty vector control and analysed GFP enrichment by flow cytometry. *RhoA* deficient pre-B cells reconstituted with RHOA were significantly enriched with GFP compared to wild-type counterpart (Figure 18B). We further confirmed the RHOA reconstitution at the expression level using a semi-quantitative PCR (Figure 18C).

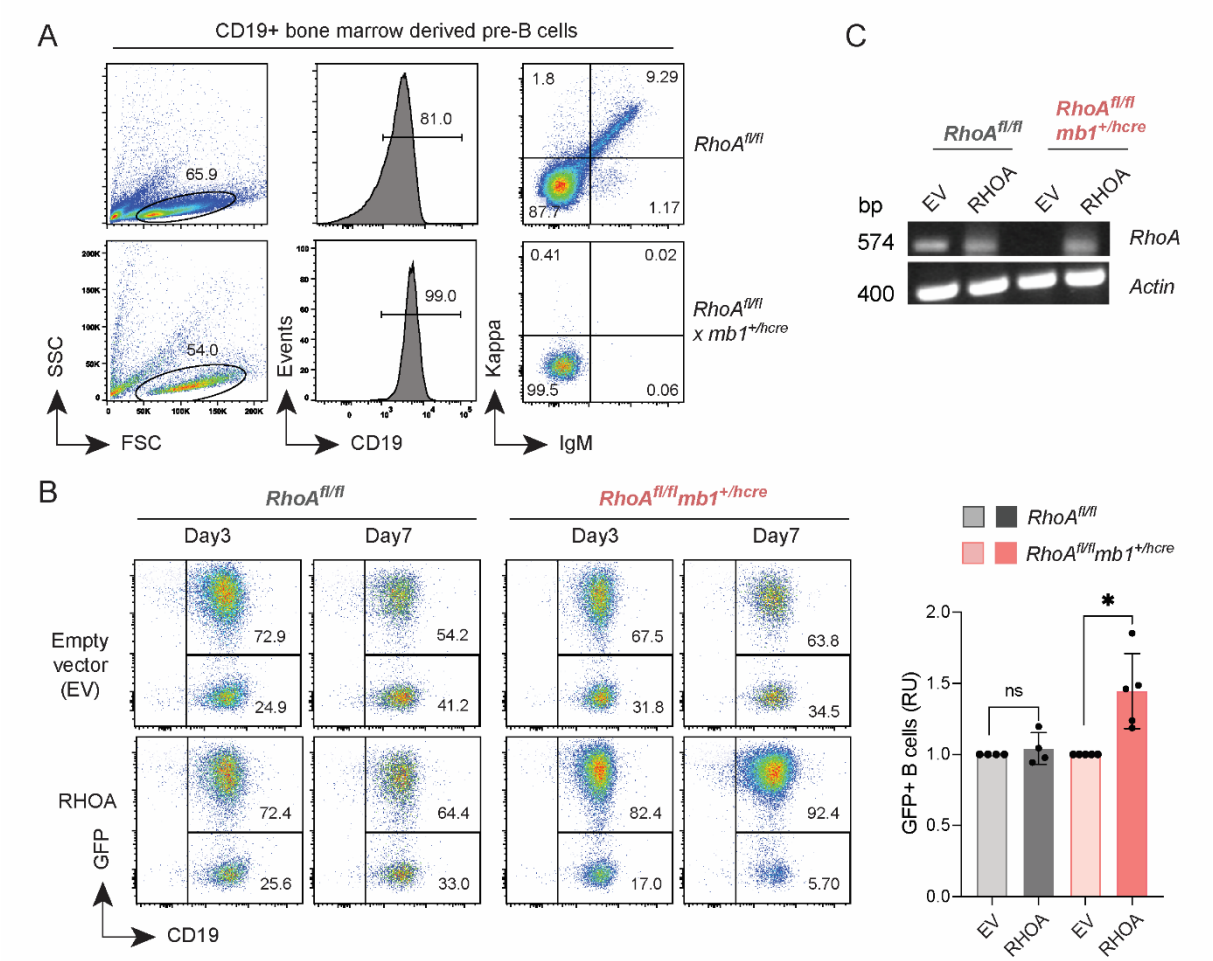

Figure 18: Overexpression of RHOA in CD19 sorted bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice: (A) Bone marrow-derived cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice were CD19 sorted using MACS and cultured in a medium with IL-7. After 3 days, the indicated markers were analyzed by flow cytometry. (B*) CD19 sorted bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice (cultured as mentioned in A) were retrovirally reconstituted with RHOA-IRES-GFP or its empty vector control and the percentage of GFP^{+} cells were measured on 3 and 7 days after transduction. Right- GFP enrichment of RHOA reconstituted cells normalized to empty vector (EV) transduced cells after normalizing to the respective percentages measured 3 days after transduction. Data are representative of at least 4 individual experiments. Statistical significance was calculated by one sample t-test. (C) CD19 sorted bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice were retrovirally reconstituted with RHOA or its empty vector control. 5 days after transduction RNA was isolated and cDNA was synthesised. *RhoA* expression was analysed at the mRNA level using semi-quantitative RT-PCR. *Actin* was used as the loading control. Data are representative of 2 individual experiments. (* is modified from Vadakumchery et al., 2022, Main Figure 5E, and F, page 8 of 19, Supplementary figure S3E, Page 3 of 11 © by the authors).

Moreover, we observed an increase in the percentage of IgM^{+}kappa^{+} cells in the *RhoA* deficient pre-B cells reconstituted with RHOA (Figure 19A). This suggests that cells reconstituted with RHOA restored their differentiation capacity and developed into BCR-expressing immature B cells. Furthermore, we found an increased expression of *Rag1* and *Rag2* in RHOA reconstituted cells compared to empty vector reconstituted *RhoA* deficient pre-B cells (Figure 19B).

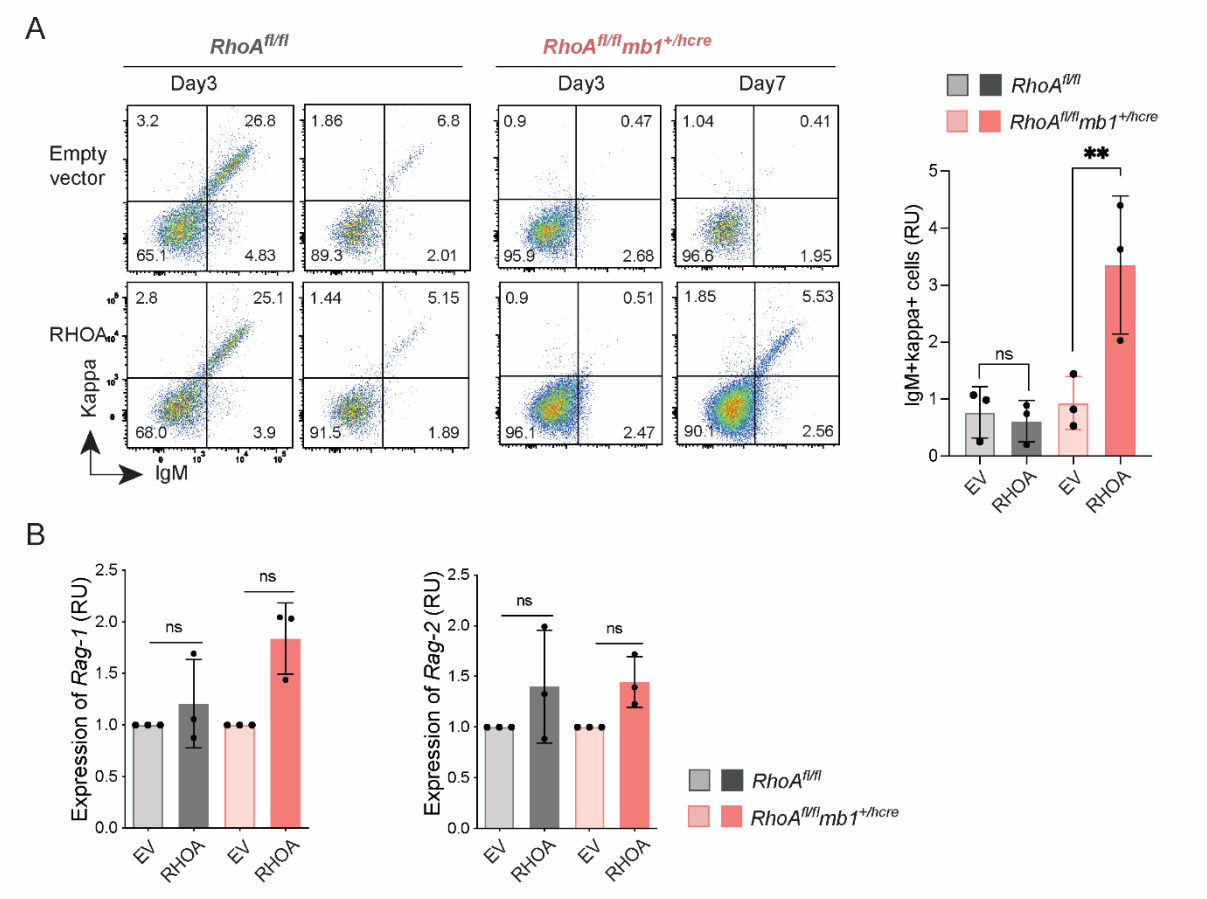

Figure 19: RHOA is required for the differentiation of pre-B cells: (A*) CD19 sorted bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice were retrovirally reconstituted with RhoA or its empty vector control. Differentiation was assessed by measuring IgM and kappa light chain expression a week after transduction. Flow cytometric analysis is representative of at least 3 individual experiments. Statistical significance was calculated using ordinary one-way ANOVA with multiple comparisons. (B*) *Rag-1* and *Rag-2* expression levels in RHOA reconstituted cells were measured in comparison to controls using quantitative RT-PCR. Results are shown as arbitrary units normalized to levels of empty vector control. Statistical analysis was done using one sample t-test. Data are representative of 3 individual experiments. (* are modified from Vadakumchery et al., 2022, Main Figure 5E, G and H, page 8 of 19, Supplementary figure S3E, Page 3 of 11 © by the authors).

Previously our group has shown that activation of *FoxO1* or *Pten* is an important requirement for *IgH* gene rearrangement. Therefore, we tested whether FOXO1 reconstitution can restore the differentiation block found in *RhoA* deficient mice. For this, we retrovirally transduced bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hCre}$ mice with FOXO1-IRES-GFP and empty vector control. However, in contrast to RHOA, FOXO1 was unable to efficiently induce light chain surface expression in *RhoA* deficient pre-B cells indicated by absence of the IgM^{+} kappa^{+} cells (Figure 20). A similar observation was found when PTEN was ectopically expressed (Vadakumchery et al., 2022). Together our data indicate that RHOA is an important factor driving the differentiation of pre-B cells.

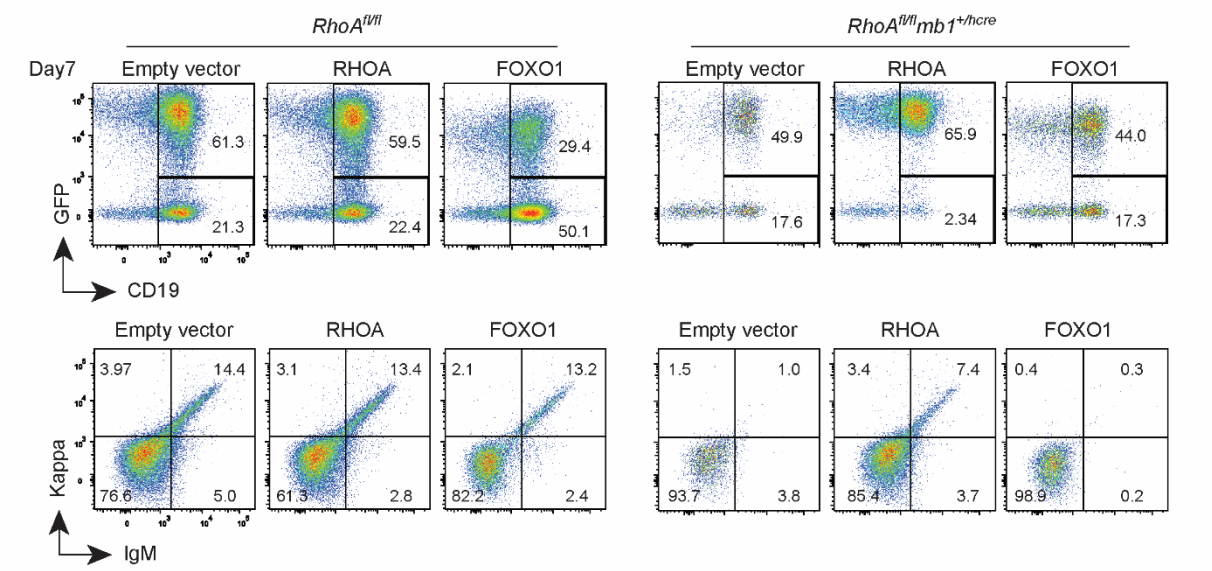

Figure 20*: Ectopic FOXO1 expression doesn't restore *in vivo* differentiation block: (A) Bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice were retrovirally reconstituted with FOXO1 or its empty vector control. GFP enrichment and differentiation (IgM and kappa expression) were measured a week after transduction. Flow cytometric analysis is representative of at least 2 individual experiments**.** (* is modified from Vadakumchery et al., 2022, Supplementary figure S3G, Page 3 of 11 © by the authors).

3.5 RHOA is important for proper development of mature B cells and generation of antibody responses

3.5.1 B cell specific deletion of *RhoA* impairs marginal zone development in the spleen

Our results show that RHOA is essential for the survival and differentiation of precursor B cells. Since *RhoA* deleted mice lack mature B cells in the periphery, we could not analyze the role of *RhoA* in mature B cell development. It has been shown that B cell-specific deletion of *RhoA* using *CD19*-Cre impaired mature B cell development in the spleen and also B cell activation factor (BAFF) mediated survival of B cells (Zhang et al., 2012). This suggests that RHOA is also important for mature B cell development. To address this, we crossed mice carrying floxed *RhoA* allele with mice expressing Cre under the control of CD21 promoter. *CD21* is expressed when immature B cells differentiate into mature B cells in the periphery (Kraus et al., 2004). We analyzed bone marrow cells from *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ by flow cytometry and as expected we found normal B cell development in these mice (Figure 21A). The total number of B cells (CD19^{+}) in the spleen of *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice was comparable to the control mice. A detailed FACS analysis of mature B cells using surface markers for CD21 and CD23 revealed that *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice have a significant reduction in marginal zone B cells (CD21^{hi} CD23^{low}) whereas the percentage of follicular B cells (CD21^{+}CD23^{+}) was not altered (Figure 21B). Furthermore, splenic cells from *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ and control mice were analysed for the expression of RHOA, RHOB, RHOC, ROCK1 and ROCK2 by western blot. A strong reduction in RHOA expression together with elevated levels of RHOB and RHOC were observed after *RhoA* deletion in mature B cells (Vadakumchery et al., 2022). This could be a

compensatory effect where knock out of one gene concomitantly increases the expression of other family members. Moreover, ROCK1 and ROCK2, the two RHO-activated kinases were also down-regulated in *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ splenic cells (Vadakumchery et al., 2022). Taken together our data demonstrate that RHOA is necessary for the proper development of marginal zones B cells in the periphery.

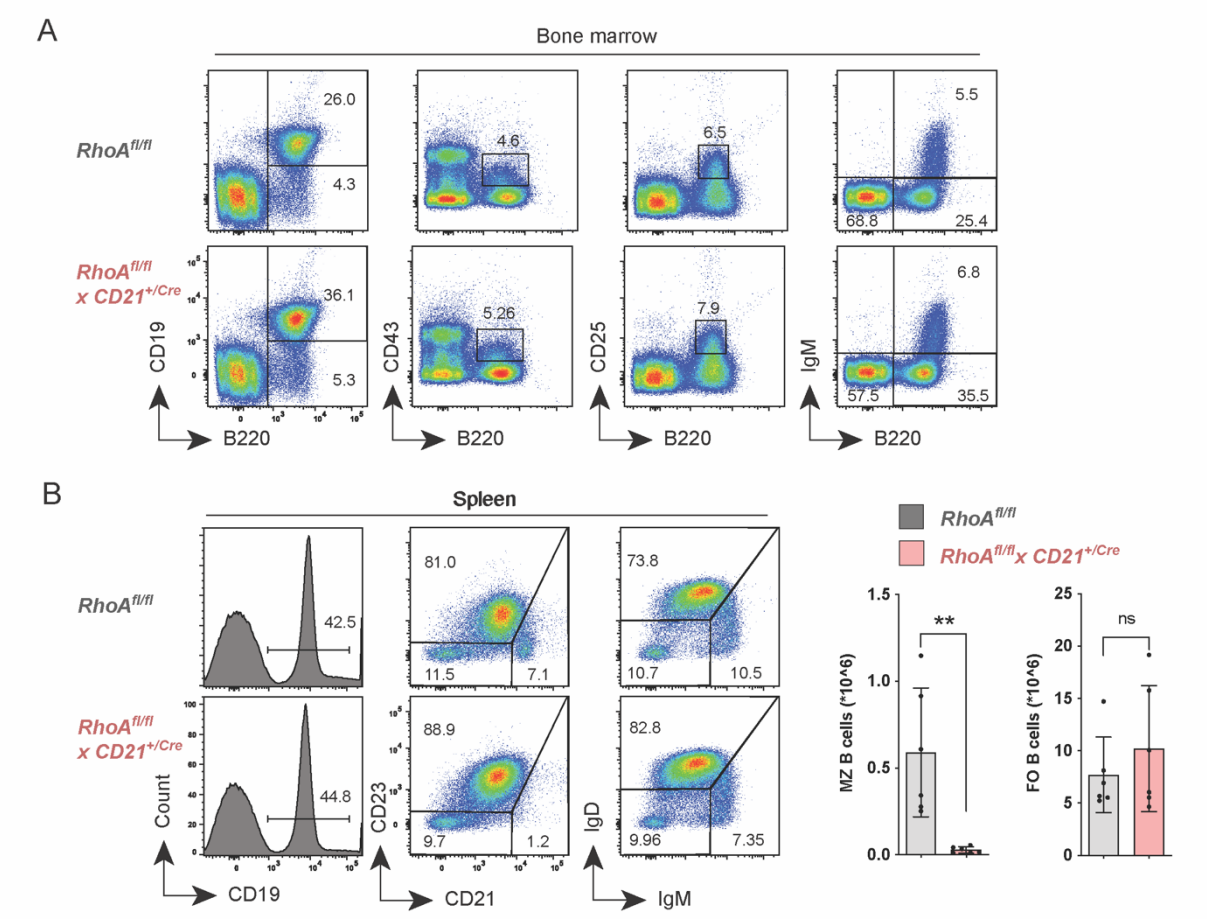

Figure 21: RHOA is required for the proper development of marginal zone B cells: (A*) Freshly isolated cells from the bone marrow of *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice were analyzed by flow cytometry for surface expression of the indicated markers. Representative data are from 2 mice per genotype. (B*) Splenic cells were isolated from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice and were analyzed by flow cytometry for the surface expression of the indicated markers. Representative data are from 6 individual mice. Absolute cell numbers of MZ and FO B cells in the spleen of *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice (n=6) were compared with control *RhoA*$^{fl/fl}$ mice (n=6). Statistical significance was calculated using Mann-Whitney U test. (* are modified from Vadakumchery et al., 2022, Main Figure 7A and B, page 8 of 19, Supplementary figure, S4A, page 4 of 11 © by the authors)

3.5.2 RHOA is required for the proliferation and differentiation of B cells into plasma cells

Rho GTPases such as RAC1, RAC2, and CDC42 are shown to be important for B cell proliferation and differentiation into antibody-producing plasma cells (Guo et al., 2009; Walmsley et al., 2003). To assess the role of RHOA in B cell proliferation and activation, we stimulated the splenic cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice with lipopolysaccharide

(LPS) and analysed the plasma cell differentiation by flow cytometry. We found that splenic cells from *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice were impaired in their ability to proliferate and form plasma cells (B220^{low} CD138^{+}) in response to LPS stimulation (Figure 22). In addition to this, we observed diminished *Blimp-1* expression after LPS stimulation in *RhoA* deficient splenic cells (Vadakumchery et al., 2022). This indicates that RHOA transduces mitogenic signals emerging from TLR4, suggesting that RHOA is required for the proliferation and differentiation of mature B cells into antibody-producing plasma cells.

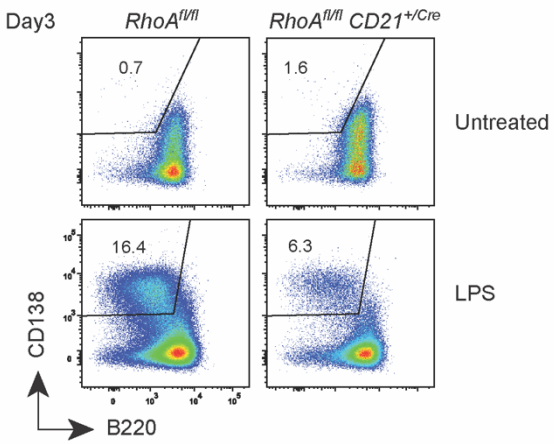

Figure 22: *RhoA* deficient B cells show impaired ability to proliferate and differentiate to plasma cells: Splenocytes from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ were left untreated or treated with LPS (2.5 µg/ml) and B220 and CD138 expression were analyzed 3 days after treatment by flow cytometry (pre-gated on CD19^{+} cells). Representative figure of 2 independent experiments.

3.5.3 Impaired immune response in *RhoA* deleted mice

We have seen that total B cells were comparable in *RhoA* deficient and the control mice. Despite having similar serum IgM titer, IgG antibody concentrations were reduced in *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice compared to control group, especially at an older age (Vadakumchery et al., 2022). To test whether antigen-specific antibody production in *RhoA* deficient B cells is compromised, *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice were immunized with T cell-dependent antigen 4-hydroxy-3-nitrophenylacetyl (24)-keyhole limpet hemocyanin (NP (24) KLH) and analysed the serum for NP-specific IgM and IgG antibodies. Serum NP-specific IgM antibodies were unaltered in *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice except for reduction on day 7 after immunization (Figure 23A). Of note, IgG titres were reduced in *RhoA* deficient mice at all measured time points before the second booster. After a booster dose injection on day 28, NP-specific IgG titer was increased in the sera of both mice (Figure 23B). We found that IgG antibodies generated after the second immunization declined rapidly in *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice compared to control (Figure 23C). These findings indicate that RHOA is necessary for generating long-lived humoral immunity.

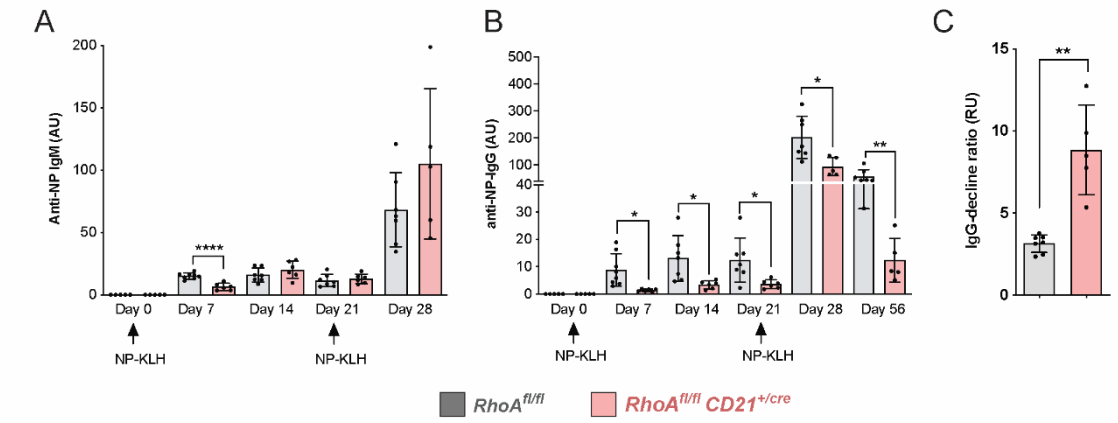

Figure 23: Impaired humoral immune response in *RhoA*$^{fl/fl}$ *CD21*$^{+/Cre}$ mice: 6-8 weeks old *RhoA*$^{fl/fl}$ (N=7) and *RhoA*$^{fl/fl}$ *CD21+/Cre* (N=8) mice were immunized with 100µg NP (24) KLH + 100µg CpG-ODN1826 in PBS or with 100µg CpG ODN1826 (control). Serum levels of NP-specific IgM (A*) and IgG (B*) antibodies were measured by ELISA on the specified days after first immunization (day 0) and booster on day 21. No NP-specific antibodies were detected in control mice injected with adjuvant (CpG-ODN1826) hence excluded from the figure. Statistical analysis was calculated using one-way ANOVA with multiple comparisons. (C*). The decline of anti-NP-IgG titers was calculated as the ratio of IgG titers at day 28 relative to day 56. Statistical significance was calculated using the Mann-Whitney U test. (* are modified from Vadakumchery et al., 2022, Main figure 8A, B and C, page 11 of 19 © by the authors).

3.6 Significance of RHOA in precursor B cell acute lymphoblastic leukaemia (BCP-ALL)

RhoA and its downstream targets ROCKs are found deregulated in many hematopoietic and non-hematopoietic malignancies (Benitah et al., 2004; Itoh et al., 1999; Lochhead et al., 2010; Sahai and Marshall, 2002). Hyperactivation of Rho kinases is observed in myeloid leukaemia's harbouring activating mutations in BCR-ABL1 and is shown to be dependent on RhoA and PI3K signalling (Singh Mali et al., 2014). This suggests a key role of RhoA in haematopoietic malignancies. SLP-65 mediated downregulation of PI3K signalling is necessary for the termination of pre-B cell proliferation and inducing its differentiation. Moreover, SLP-65 deficient pre-B cells remain in the proliferative stage and this further lead to cellular transformation and subsequently pre-B-cell leukaemia. About 7% of SLP-65 deficient mice are prone to leukaemia, suggesting that signalling factor/s that link SLP-65 to PI3K signalling is important and need further investigation, especially in B cell precursor leukaemia (BCP-ALL). Our findings show that RhoA is a key component in B cell development which connects SLP-65 activation to PI3K/PTEN signalling pathway. This led us to investigate the role of RHOA in BCP-ALL.

3.6.1 RHOA is required for the survival of BCR-ABL1 transformed pre-B cells

To investigate the role of RHOA signalling in BCP-ALL, we first determined the expression of *RhoA* in BCP-ALL samples. These samples were propagated as xenografts in immunodeficient mice and included both BCR-ABL1 positive and negative karyotypes (Table 10). As a control,

we used the BCP-ALL cell line, EU-3 with characteristic t(1;19)(q23; p13) translocation which leads to E2A-PBX1 fusion (Kamps and Baltimore, 1993; Storti and Moorman, 2016). We found that RhoA is ubiquitously expressed in all BCP-ALL samples (Figure 24). Moreover, RhoA shows a 1.3% alteration in leukaemia and lymphomas mainly by inactivating mutations or deletions compared to RhoB, RhoC, and effector kinases (Cerami et al., 2012; Gao et al., 2013). Our result suggests an important role of RHOA in the BCP-ALL.

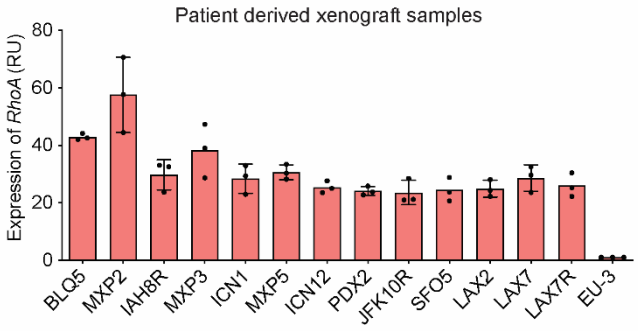

Figure 24: RhoA expression in BCP-ALL subtypes: RhoA expression levels were determined by quantitative RT-PCR in 13 PDX BCP-ALL subtypes. Results are shown as relative units (RU) normalized to the expression levels measured in the 697 (EU-3) cell line. Data are representative of 3 independent experiments.

Our data show that RHOA is indispensable for pre-B cell development (Figures 12 and 13). This led us to further investigate the role of RHOA in pre-B cell malignancies, especially in Philadelphia chromosome-positive (Ph^+) BCP-ALL which is a most frequent (25-30 %) ALL subtype and is characterised by the BCR-ABL1 fusion protein (Schultz et al., 2014). Over 90% of chronic myeloid leukaemia (CML) patients harbour this BCR-ABL1 protein (Kabarowski and Witte, 2000). Previously we have seen that B cell precursors require RHOA for their survival and enrichment in IL-7-dependent cultures (Figure 14). Therefore, in our study, we first investigated whether RHOA is necessary for the generation of BCR-ABL1 transformed ALL cells. To understand this, we transduced bone marrow cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hCre}$ mice immediately after isolation with a BCR-ABL1-IRES-GFP retroviral vector or its empty control. BCR-ABL1 transformed pre-B cells proliferate independently of IL-7 (Abdelrasoul et al., 2020). We analysed GFP enrichment (a marker for BCR-ABL1 transformation) 3 days after IL-7 withdrawal using flow cytometry. Our results show that bone marrow-derived cells from *RhoA* deficient mice could give rise to BCR-ABL1 induced ALL (Figure 25A). Since RHOA, RHOB, and RHOC are highly homologous GTPases which share 88% sequence homology (Schaefer et al., 2014), we tested whether the RhoA deficiency in BCR-ABL1 transformed BCP-ALL cells is compensated by the expression of other Rho isoforms. To test this, we analysed the expression of RhoA, RhoB, and RhoC at mRNA level in BCR-ABL1 transformed *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hCre}$ cell lines using semi-quantitative PCR. We found that BCR-ABL1 transformed *RhoA* deficient cell line upregulates the expression of the RhoB (Figure 25B). Taken together, our data suggest that RhoA is not necessary for the BCR-ABL1 mediated transformation of precursor B cells and *RhoA* deficient B cells upregulate the expression of RhoB to compensate for the effect of RhoA deletion.

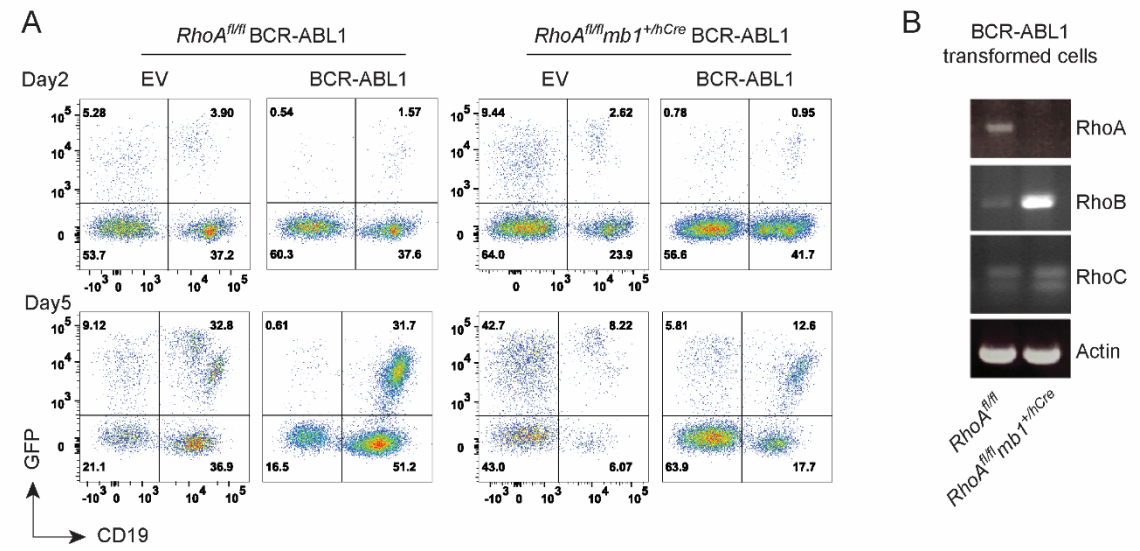

Figure 25: RhoA is not required for BCR-ABL1 mediated transformation of B cell precursors: Left- BM-derived pro/ pre-B cells from *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ mice were transduced with either an empty vector (EV) or BCR-ABL1 expression vector. The enrichment of CD19^{+}GFP^{+} cells were determined 2 days (Day 2) after transduction and 3 days after IL-7 withdrawal (Day 5). The results are representative of 2 independent experiments. Right- RNA was isolated and cDNA was synthesised from BCR-ABL1 transformed *RhoA*$^{fl/fl}$ and *RhoA*$^{fl/fl}$ *mb1*$^{+/hcre}$ cell lines. RhoA, RhoB, and RhoC expression were analysed using semi-quantitative PCR. Actin was used as the loading control. Representative figure from 2 independent experiments.

To gain further insight into the role of RHOA in the survival of BCR-ABL1 transformed cells, we further transduced *RhoA*$^{fl/fl}$ BCR-ABL1 transformed cells with a retroviral expression vector encoding 4-OHT inducible form of Cre recombinase (Cre ER^{T2}) or its empty vector control (ER^{T2}). Cells were treated with 4-OHT or its control ethanol for 3 days to induce *RhoA* deletion and the viability of the treated cells was analysed by flow cytometry. Inducible deletion of *RhoA* in BCR-ABL1 transformed cells led to a significant reduction of viable cells in Cre ER^{T2} cells after the addition of tamoxifen (Figure 26A and B). Moreover, RhoA is shown to be essential for leukaemia development and its progression in *in vivo* conditions (Vadakumchery et al., 2022). Small GTPases, like RHOA, often function downstream of G-protein coupled receptors, such as C-X-C family chemokine receptor 4 (CXCR4), which is shown to be essential for the development and migration of B cells (Bos et al., 2007). To assess the significance of the RhoA/ROCK signalling pathway in the migration of Ph^{+} BCP-ALL cells, we treated bone marrow-derived *RhoA*$^{fl/fl}$ BCR-ABL1 transformed cells with cell-permeable Rho-specific inhibitor (CT04, 2 µg/ml) and membrane permeable ROCK inhibitor (H-1152, 5 µM) along with untreated cells. Previously our group had shown that in Ph^{+} BCP-ALL, BCR-ABL interacts with CXCR4 and this interaction brings IL-7R signalling machinery near BCR-ABL1 thereby enabling its activation and pre-B cell transformation (Abdelrasoul et al., 2020). We found that the inhibition of RHOA and ROCK significantly reduced cell migration toward CXCL12, the ligand for the chemokine receptor CXCR4 (Figure 26C). Taken together, these findings suggest that RHOA is important for the survival and migration of Ph^{+} BCP-ALL cells.

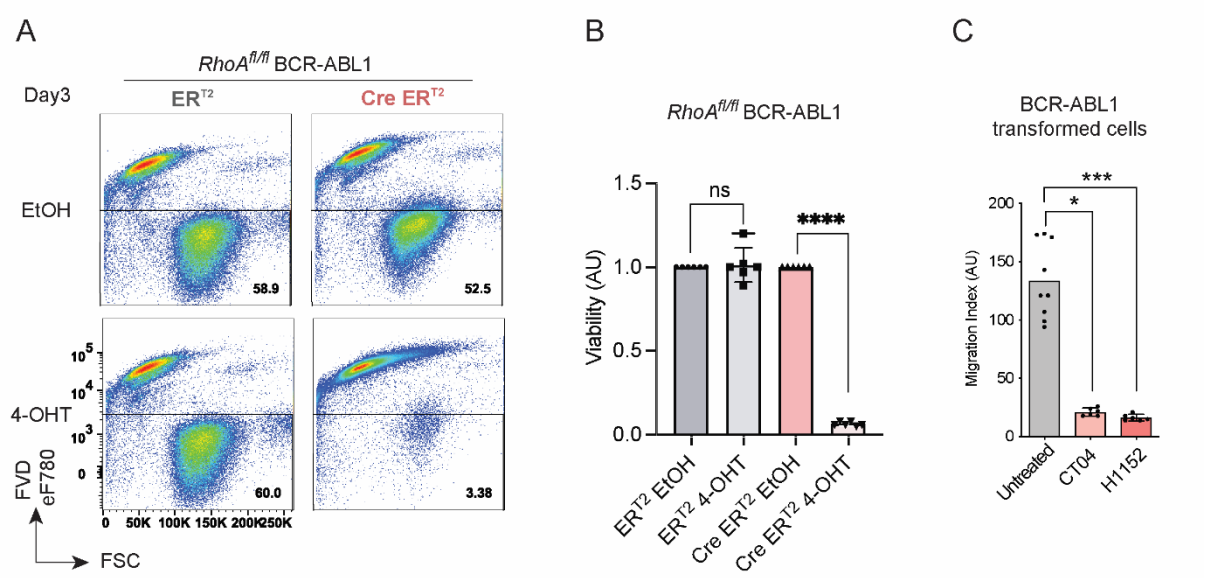

Figure 26: RHOA is essential for the survival and migration of BCR-ABL1 transformed pre-B cells: (A*) Bone marrow-derived cells from *RhoA*$^{fl/fl}$ mice were retrovirally transduced with BCR-ABL1 and subsequently transduced with either ER^{T2} or Cre-ER^{T2}. Cells were treated with 2 µM 4-OHT or the solvent ethanol (EtOH) to induce Cre recombination. The viability of the cells was measured 3 days after 4-OHT treatment by flow cytometry. (B*) Quantification of viable cells after 4-OHT treatment in comparison with the controls. Statistical significance was calculated using one-sample t-test. Data are representative of 6 individual experiments. (C*) Pre-B cells transformed with BCR-ABL1 were treated with Rho-specific inhibitor (CT04, 2µg/ml), and ROCK inhibitor (H1152, 5µM) or with the vehicle for 24 hours and cells were subjected to migration towards 100 ng/ml CXCL12 for 16 hours. The cells in the lower chamber were counted using trypan blue. Statistical significance was calculated using the Mann-Whitney U test. (* are modified from Vadakumchery et al., 2022, Main figure 6A and B, page 9 of 19 © by the authors).

Hyperactivation of Rho GTPase pathways is observed in myeloid leukaemia and myeloproliferative diseases harbouring activating mutations like BCR-ABL1. Moreover, RhoA/ROCK inhibitors treatment resulted in growth inhibition and apoptosis of myeloid leukemic cells (Singh Mali et al., 2014). Therefore, in our study, we further investigated the effect of RHOA inhibition on the growth of BCR-ABL1 transformed cells. To inhibit RhoA, we treated wild-type pre-B cells transformed with BCR-ABL1 with different concentrations of Rho-specific inhibitor, CT04 for 48 hours. As controls, we used untreated cells. We found that the RhoA inhibitor repressed the growth of transformed cells in a dose dependent manner (Figure 27A). We further assessed whether ROCK inhibitors also suppress the growth of BCR-ABL1 transformed cells. For this, we treated transformed cells with increasing concentrations of ROCK inhibitor (H-1152) for 48 hours and cell viability was assessed by flow cytometry. Following, RhoA inhibition results, we observed a dose-dependent growth suppression in BCR-ABL1 transformed cells after H1152 treatment. Significant reductions in proliferation were only observed at a higher concentration of H1152 (Figure 27B). Taken together, these findings suggest that RhoA/ROCK signalling pathway is important for the survival of BCR-ABL1 transformed BCP-ALL cells.

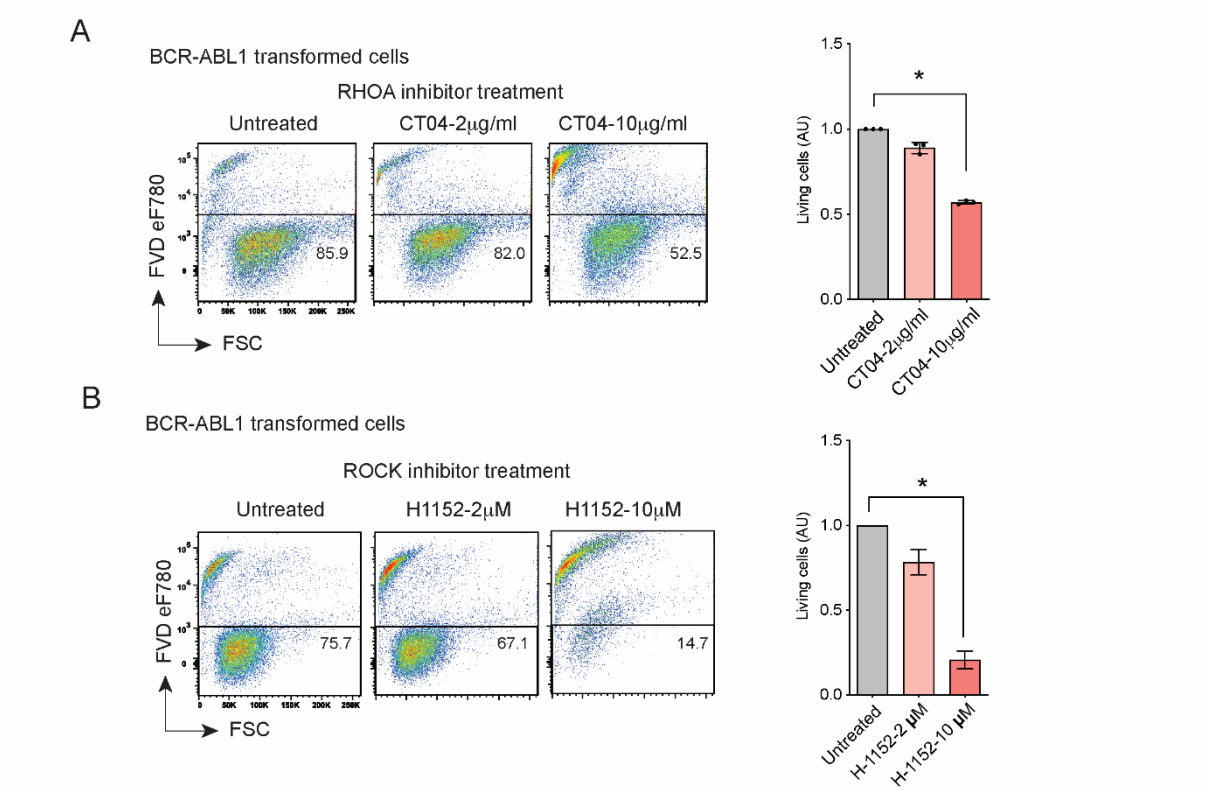

Figure 27: RhoA/ROCK inhibitors suppress the growth of BCR-ABL1 transformed cells: BCR-ABL1 transformed cells were treated with the indicated concentrations of the Rho-specific Inhibitor-CT04 (A), Rock inhibitor-H1152 (B) and the percentage of viable cells was determined 48 hours after treatment using flow cytometry. Statistical significance was calculated using the Mann- Whitney U test. Data are representative of 3 independent experiments.

4 Discussion

4.1 Small GTPase RHOA connects SLP-65 activation to PI3K signalling and is important for early and mature B cell development

4.1.1 RHOA, the novel interaction partner for SLP-65, connects SLP-65 activation to PI3K-PTEN signalling

The Phosphoinositide 3-Kinase (PI3K) pathway is an important signalling axis activated upon pre-BCR/BCR engagement which controls the proliferation, differentiation, and survival of B cells (Abdelrasoul et al., 2018; Okkenhaug and Vanhaesebroeck, 2003). The adaptor protein SLP-65, which is activated upon pre-BCR/BCR engagement, downregulates PI3K signalling and thereby induces pre-B cell differentiation. In this study, we have shown how SLP-65 modulates PI3K signalling and induces differentiation of developing B cells. Our studies in human Burkitt lymphoma-derived cell line DG75EB that overexpresses HA tagged RHOA (DG75EB/HA-RhoA) led us to identify Rho guanine nucleotide exchange factor 2 (*ARHGEF2*, also known as GEF-H1), as the novel interaction partner of SLP-65. GEF-HI is already known to be the activator of the Rho family of GTPases including RHOA, RHOB and RHOC (Bos et al., 2007). Moreover, RHOA of the Rho family of small GTPases is shown to be essential for B cell development (Zhang et al., 2012). In our study, the Proximity ligation assay (PLA) results show that SLP-65 and RHOA are in close proximity and their interaction takes place without BCR stimulation in B cells. Importantly, we could also show that BCR stimulation and subsequent SLP-65 activation immediately recruit RHOA to activated SLP-65. These results collectively indicate that RHOA can interact with SLP-65 either directly or indirectly through GEF-H1. Furthermore, using an inducible ER^{T2} SLP-65 experimental system we have shown that inducing SLP-65 activity results in RHOA activation. Taken together, our data suggest that RHOA and GEF-H1 are recruited to the SLP-65 signalosome and this interaction leads to the activation of RHOA.

The lipid phosphatase PTEN (phosphatase and tensin homolog) is a catalytic antagonist of PI3K signalling which dephosphorylates PIP_3 to PIP_2 (Leslie and Downes, 2002; Maehama and Dixon, 1998). RHOA activation leads to the induction of its effector molecule, Rho-associated coiled-coil containing kinase (ROCK) (Amano et al., 2010; Nakagawa et al., 1996). It was previously reported that RhoA/ROCK pathway regulates the intracellular localization of PTEN in leukocytes and human embryonic kidney cells (Li et al., 2005; Sanchez et al., 2005). We observed that inhibition of RHOA in pre-B cells led to the activation of PI3K signalling shown by increased phosphorylation of AKT. We also saw a small but significant increase in PTEN protein, which can be due to the increased accumulation of PTEN in the cytosol which has failed to get recruited to the membrane in the absence of RHOA. By using the inducible TKO-EST cell system we have shown that SLP-65 induction leads to the intracellular localization of PTEN which is used as a read-out for PTEN activity. Furthermore, RHOA inhibition prevented PTEN clustering and subsequent recruitment towards the membrane (Vadakumchery et al., 2022). Taken together our data indicate that activation of RHOA by SLP-65 leads to induction of this RHOA/PTEN pathway thereby counteracting PI3K signalling. This makes RHOA to be the key signalling molecule that links SLP-65 activation and PI3K pathway down-regulation.

4.1.2 RHOA is essential for survival and differentiation of early B cells

We have further confirmed the indispensable role of RHOA in early B cell developmental stages using the *mb1*$^{+/hCre}$ mouse model, which enables *RhoA* deletion even at an early developmental stage (pro-B cell stage). Inactivation of *RhoA* at an early stage of B lymphopoiesis led to the complete block of B cell development at the pro-B cell stage, with no mature B cells in the periphery. SLP-65 regulates pre-B cell differentiation by regulating the PI3K-AKT-FOXO1 pathway (Herzog et al., 2008). The phenotype we observed in *RhoA*$^{fl/fl}$ *mb1*$^{+/hCre}$ resembles the phenotype of Pten$^{fl/fl}$ or *FoxO1*$^{fl/fl}$ mice crossed to mb1-cre, which further confirms our *in vitro* observation that RHOA links SLP-65 function to the regulation of PI3K signalling (Alkhatib et al., 2012; Dengler et al., 2008; Setz et al., 2018). It has been shown that deletion of *RhoA* in hematopoietic stem cells using Mx-Cre leads to an increase in common lymphoid progenitor (CLP) together with a reduction of Pro/Pre/Immature B cell compartments in the bone marrow (Zhang et al., 2012). This signifies the role of RHOA in progenitor/pre-B cell differentiation. Pro-B cell developmental blocks occur mainly due to the defect in IL-7R signalling resulting in reduced proliferation and cell death (Corcoran et al., 1996). It has been shown that *FoxO1* deficient pro-B cells showed defective IL-7-dependent proliferation and survival due to impaired IL-7R expression (Alkhatib et al., 2012; Dengler et al., 2008). Furthermore, our data show that *RhoA* deficient B cells also showed defective IL-7-dependent proliferation and survival. Similarly, male germ cell *Rac GAP* (mgcRacGAP) deficient pro-B cells exhibited similar proliferation and survival defects as seen in *RhoA* and *FoxO1* deficient pro B cells. Furthermore, ectopic expression of the anti-apoptotic factor, Bcl-xL rescued these cells from cell death (Kawashima et al., 2000; Yamada et al., 2006).

By using an inducible *RhoA* deletion system we have shown that *RhoA* is essential for the survival and differentiation of pre-B cells. Moreover, we have observed that RHOA is required for proper PTEN expression and regulation of the PI3K signalling pathway in pre-B cells. *Ig* rearrangement is highly regulated by PI3K (Herzog et al., 2009; Werner et al., 2010). B cell developmental block observed in *Pten* or *FoxO1* deleted pro-B cells is primarily due to defects in *Ig* gene rearrangement (Alkhatib et al., 2012; Amin and Schlissel, 2008; Dengler et al., 2008; Setz et al., 2018). Notably, *in vitro* reconstitution of RHOA in *RhoA*-deficient pro-B cells restored *Ig* rearrangement which ultimately led to the differentiation of these cells into BCR expressing immature B cells. Surprisingly, we observed that reconstitution of PTEN or FOXO1 could not overcome the differentiation block observed in *RhoA*-deficient pro-B cells indicating the unique role of RHOA in driving the differentiation of early B cells.

4.1.3 RHOA is important for the development and antibody response of mature B cells

Our study indicates that RhoA is also required for the proper development of mature B cells in the periphery. Using the *CD21*$^{+/Cre}$ mouse model, we have shown that *RhoA* deletion at later stages of B cell development led to the complete absence of marginal zone (MZ) B cells. B cell -specific deletion of *RhoA* using CD19-Cre impaired mature B cell development in the spleen (Zhang et al., 2012). Furthermore, *RhoA* deficiency led to reduced BAFF receptor expression, loss of BAFF-mediated activation of AKT and survival of B cells (Zhang et al., 2012). Studies have shown that other Rho GTPases also play a critical role in mature B cell development. B cell-specific deletion of Cdc42 results in a significant reduction in the peripheral B cell

population (Gerasimcik et al., 2015; Guo et al., 2009). Similarly, *Rac2* deficient mice also showed a reduction in MZ and B1-b cells (Croker et al., 2002). p115 RhoGEF-H1/ARHGEF1, an upstream activator of RHOA is shown to be essential for marginal zone B cell homeostasis (Girkontaite et al., 2001). Dock11, a GEF for Rho GTPases is also shown to be important for splenic MZ B cell development (Nishikimi et al., 2005). Thus, our data along with other published results provide evidence for the involvement of migration pathways in the generation and maintenance of marginal zone B cells.

MZ B cells express polyreactive BCRs as well as higher levels of Toll-like receptors (TLRs) and respond to low doses of T-independent polyclonal antigens faster than conventional FO B cells (Cerutti et al., 2013; Oliver et al., 1997). This dual engagement of BCRs and TLRs by conserved microbial molecules such as lipopolysaccharides or peptidoglycan enables MZ-B cells to generate low-affinity first-line antibody responses (Bendelac et al., 2001; Martin et al., 2001). We have observed *RhoA* deficient mice showed impaired ability to proliferate in response to LPS stimulation and differentiate into antibody-producing plasma cells *in vitro*. In contrary, no such proliferation defects were observed after CpG stimulation in *RhoA* deficient B cells (Vadakumchery et al., 2022). This indicates that RHOA is required for transducing proliferation signals emerging through TLR4 but is dispensable for TLR9. The attenuated proliferative response of *RhoA* deficient B cells to LPS is possibly due to the significant reduction of MZ B cells observed in *RhoA* deficient mice. Similarly, *Cdc42* deficient B cells also showed impaired response to LPS stimulation whereas Rac2 deficient B cells proliferated normally despite having reduced MZ B cells (Croker et al., 2002; Guo et al., 2009). The reduction in the generation of plasma cells could be due to a reduced survival signal. Indeed, *RhoA* deficiency was shown to completely block BAFF-mediated AKT activation and subsequent B cell survival (Zhang et al., 2012). Similarly, Cdc42 also regulates BCR and BAFFR signalling mediated B-cell survival and proliferation (Guo et al., 2009). Furthermore, our data also show that B lymphocyte-induced maturation protein (BLIMP-1); the master regulator of plasma cell differentiation (Shaffer et al., 2002; Shapiro-Shelef et al., 2003) was reduced after LPS stimulation in *RhoA* deficient B cells (Vadakumchery et al., 2022). This shows that *RhoA* deficient B cells cannot be committed to terminal differentiation probably due to deregulated PI3K signalling (Setz et al., 2018, 2019). MZ B cells are shown to upregulate the expression of T cell co-stimulatory molecules in the earliest stage of *in vivo* immune response (Martin and Kearney, 2002; Oliver et al., 1997). We observed comparable expression of the activation markers CD86 and CD69 in response to different stimuli in *RhoA* deficient and control B cells (Vadakumchery et al., 2022). This could be due to functional redundancy by other Rho GTPase family members such as Cdc42, Rac1/2, RhoB and RhoC. Further studies are necessary to see if other RHO family members also participate in the SLP-65/PTEN pathway to regulate B cell differentiation and function. Taken together, our data suggest that the RHOA function is essential for the proper selection and regulation of B cells.

FO B cells upon T-cell dependent antigen exposure migrate to follicles and form the GC (Kerfoot et al., 2011; Kitano et al., 2011). In *RhoA* deficient mice we have observed no significant reduction in FO B cells. Conversely, we have seen significant reduction in IgG serum titre in *RhoA* deficient mice without immunization (Vadakumchery et al., 2022). We also observed a significant reduction of NP specific IgG but not IgM antibodies in *RhoA* deficient

mice immunized with NP(24)KLH, a T cell dependent antigen. Our results indicate that RHOA is important for GC reaction and generation of high affinity antibodies of IgG types. In fact, *Cdc42* deficient B cells showed reduction in both NP- specific IgM and IgG1 antibodies (Guo et al., 2009). This discrepancy in NP-specific IgM could be due to the reduction in MZ and FO B cells found in *Cdc42* deficient mice. Furthermore, *Dock 10* deficient mice showed lower IgG response to NP-KLH *in vivo* (Gerasimcik et al., 2017). *Baff-R* deficient mice exhibited reduced TD Ag-specific IgG1 primary and secondary immune response whereas IgM was only slightly reduced (Sasaki et al., 2004; Vora et al., 2003). Our results have shown the significance of RHOA in regulating PTEN expression and subsequent PI3K signalling in pre-B cells. GC B cells exhibit altered PI3K-AKT signalling with increased activation of FoxO1, a key factor regulating GC B cell selection (Dominguez-Sola et al., 2015; Luo et al., 2018; Sander et al., 2015). Moreover, it has been shown that GC B cells express increased level of PTEN, which negatively regulates PI3K signalling (Luo et al., 2019). We propose that the reduced immune response (particularly the generation of IgG antibodies) might be due to defective activation of PTEN in *RhoA* deficient B cells. Previously, it has been shown that, GEF-H1is required for the confinement of B cells within GC (Bouafia et al., 2019). Recent report has shown that B cell specific ablation of *Rock2* leads to impaired GC formation and defective humoral immune response. Accordingly, they found that ROCK2 restrained AKT activation and promoted the transcriptional activity of FoxO1 (Ricker et al., 2020). In addition, CXCR4 expression plays a critical role in the maintenance of GC architectural polarity and is mainly regulated by FoxO1 (Allen et al., 2004; Nie et al., 2004; Sander et al., 2015). GC B cells show extensive interzonal migration and this motility is regulated by actin polymerization which depends on Rho GTPases (Gerasimcik et al., 2015). We have found that *RhoA* deficient B cells showed impaired migration toward CXCL12, the ligand for CXCR4 (Vadakumchery et al., 2022). This suggests that B cell migration depends on RhoA and disruption of RhoA signalling pathway could destroy GC architecture, B cell migration, and the humoral responses. In splenic B cells, *RhoA* deficiency led to reduced expression of BAFF receptor (Zhang et al., 2012). BAFF/BAFF-R mediated chemotaxis to CXCL12 in primary human B cells shown to be dependent on PI3K/AKT pathway which subsequently leads to RhoA/ROCK activation (Badr et al., 2008). Taken together, our results show that RHOA play a significant role in the generation of proper humoral immune response.

4.2 RhoA is required for the survival and migration of BCR-ABL1 transformed ALL cells

Philadelphia chromosome (Ph) positive acute lymphoblastic leukaemia (ALL) is characterised by a reciprocal translocation between the long arms of chromosomes 9 and 22 (t(2;22)(q34;q11)) leading to the formation of the BCR-ABL1 fusion protein. This translocation occurs in about 5% of children and 20-30% of adults with ALL and is associated with poor prognosis (Faderl et al., 1998; Hantschel, 2012). With the development of tyrosine kinase inhibitors which block the activity of BCR-ABL1, the treatment outcome of Ph^+ BCP-ALL has drastically improved (Schultz et al., 2014). However, the emergence of drug-resistant clones during treatment is a major problem that leads to relapse. It is presumably due to the involvement of growth factors that are present in the microenvironment that protects the resistant clones which eventually develop into leukaemia initiating cells (LIC). In addition, understanding the

molecular mechanism of BCR-ABL1 transformation is of pivotal importance which enables the development of novel inhibitors and alternative therapeutic strategies.

Rho GTPases are crucial signal transducing molecules that regulate cell migration, proliferation (Singh Mali et al., 2014). The aberrant expression or function of GTPases and their downstream targets leads to the growth, progression and metastasis of various cancers (Rath and Olson, 2012). RhoA and its downstream effector kinases are found deregulated in many hematopoietic malignancies (Benitah et al., 2004; Itoh et al., 1999; Lochhead et al., 2010; Sahai and Marshall, 2002). Inactivating mutations in RhoA or its activators are found in B and T cell lymphomas (Manso et al., 2014; O'Hayre et al., 2016; Sakata-Yanagimoto et al., 2013). We observed a broad range of RHOA expression in different BCP-ALL subtypes. Our results show that RHOA is not necessary for BCR-ABL1 mediated ALL generation. Furthermore, we have seen that *RhoA* deficient BCR-ABL1 transformed cells upregulate the expression of RhoB. This could be due to the compensatory mechanism that rescues the effect of RhoA deletion suggesting certain degree of redundant function of these proteins. Our data also show that RHOA is essential for the survival of BCR-ABL1 transformed pre-B cells. This goes in line with previous studies in chronic myeloid leukemia (CML) where BCR-ABL1 oncogene activates RhoA through its RhoGEF activity and is required for the transformed phenotype (Sahay et al., 2008). Further experiments should be performed to identify the molecular mechanism by which RHOA induces BCR-ABL1 transformation and whether it is through the identified SLP65-PTEN-PI3K pathway. We observed that the leukemic cells require RHOA and its downstream kinases for their migration. Constitutive activation of the ROCK is found in leukemic cells harbouring activating mutation in KIT, FLT-3 and BCR-ABL1. Furthermore, the inhibition of RhoA and ROCK resulted in growth inhibition and survival of the leukemic cells *in vitro* (Mali et al., 2011). Our data indicate that RhoA/ROCK signalling pathway is important for the survival of Ph^+ BCP-ALL cells. Furthermore, we also confirmed that RHOA deficiency prevents BCP-ALL development and leukemic progression *in vivo* (Vadakumchery et al., 2022). Taken together, our results suggest the significant therapeutic relevance of RHOA/ROCK inhibitors in the treatment of BCP-ALL. Can RhoA/ROCK inhibitors be used in the tyrosine kinase driven malignancies in combination with tyrosine kinase inhibitors (TKIs)? This question remains to be further investigated.

5 Summary

Early B cell development is highly regulated by the signals emerging from the pre-BCR which activates many signalling pathways controlling the proliferation, survival, and differentiation of developing B cells. One of the important signalling pathways that are activated downstream of pre-BCR/BCR is PI3K signalling which controls the proliferation as well as the survival of developing B cells. In early B cells, SLP-65 downregulates the PI3K signalling and subsequently induces pre-B cell differentiation. In this study, we have investigated the molecular mechanism by which SLP-65 regulates the PI3K signalling and induces the differentiation of developing B cells. Our results show that SLP-65 interacts with small GTPase RHOA and its activator, GEF-HI. Furthermore, SLP-65 mediated activation of RHOA leads to the induction of PTEN, which negatively regulates PI3K signalling. B cell-specific inactivation of *RhoA* using $mb1^{+/hcre}$ leads to the complete block of B cell development at the pro B cell stage, indicating the essential role of RHOA in early B lymphopoiesis, especially in the pre-B cell differentiation. *In vivo* differentiation block was restored with RHOA reconstitution in *RhoA* deficient B cell progenitors confirming the key role of RHOA in B cell development. Moreover, we show that RHOA is essential for the *in vitro* and *in vivo* survival of early B cells. In addition to its essential role in early B cell development, our data suggest that RHOA is necessary for peripheral B cell development and the generation of antibody-producing plasma cells. The IgG response to T-cell-dependent antigen was significantly lower when *RhoA* was specifically deleted in B cells. Taken together our data show that RHOA is indispensable for B cell development and function, which connects pre-BCR/BCR mediated SLP-65 activation to PI3K-PTEN signalling. In addition, to the critical role of RHOA in B cell development and differentiation, we found that RhoA is necessary for the survival of BCR-ABL1 transformed precursor B cells. We observed that BCR-ABL1 bearing ALL cells were sensitive to RhoA/ROCK inhibitors. This suggests the relevance of targeting RhoA/ROCK signalling pathways in the treatment of BCP-ALL.

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Appendix

Table 10: Characteristics of patient derived B-ALL samples (Shojaee et al., 2016)

Number	BCP-ALL Xenografts	Diagnosis	Stage	Type
1	BLQ5	Ph+ pre-B-ALL	Relapse (Imatinib)	BCR-ABL1 (T3151)
2	IAH8R	Ph+ pre-B-ALL	Relapse (Dasatinib)	BCR-ABL 1(F359V)
3	ICN1	Ph+ pre-B-ALL	Diagnosis	BCR-ABL1
4	ICN12	Pre-B-ALL	Diagnosis	TCP3-PBX1
5	JFK10R	Pre-B-ALL	Relapse to JFK10	
6	LAX2	Ph+ pre-B-ALL	Relapse (Imatinib)	BCR-ABL (T3151)
7	LAX7	Pre-B-ALL	Diagnosis	
8	LAX7R	Pre-B-ALL	Relapse to LAX7	KRAS ^{G12V}
9	MXP2	Ph+ pre-B-ALL	Diagnosis	BCR-ABL1; PAX5 Deletion
10	MXP3	Ph+ pre-B-ALL	Diagnosis	BCR-ABL1; PAX5 Deletion
11	MXP5	Ph+ pre-B-ALL	Diagnosis	BCR-ABL1; PAX5 Deletion (Exon 2-6)
12	PDX2	Ph+ pre-B-ALL	Diagnosis	BCR-ABL1
13	SFO5	Pre-B-ALL	Diagnosis	Normal karyotype

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Synergism between IL-7R and CXCR4 drives BCR-ABL induced transformation in Philadelphia chromosome-positive acute lymphoblastic leukemia. -Nature communications, (2020). - Co-author.

IL-7R is associated with CNS infiltration and relapse in pediatric B-cell precursor acute lymphoblastic leukemia. Letters to Blood (2018). - Co-author.