INPP4's Role in the Regulation of BCR Signaling and B Cell Metabolic Activity

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ABSTRACT

Inositol polyphosphate 4-phosphatase (INPP4) regulates the PI3K signaling pathway by hydrolysis of the D-4 phosphate of phosphatidylinositol (3,4) bisphosphate (PI(3,4)P2) to produce PI(3)P1. The PH-domain containing proteins Akt and Btk differentially bind to PI(3,4)P2 and PI(3,4,5)P3 produced from PI3K activation, and each contribute to different and equally important downstream signaling pathways activated during B cell antigen receptor (BCR) signaling. However, there is limited understanding of how PI(3,4)P2 hydrolysis affects these specific downstream signaling pathways, and no studies have examined how INPP4 aids in regulation of lymphocyte activation. It is hypothesized that INPP4 regulates BCR signaling and cell metabolic activity via the hydrolysis of PI(3,4)P2.

Various biochemical and analytical techniques (confocal microscopy and ECAR assays) were utilized to determine how PI(3,4)P2 depletion by INPP4A overexpression affects the regulation of Akt phosphorylation, Btk/PLCγ phosphorylation and calcium flux, and B cell metabolism during BCR stimulation. INPP4A WT overexpression in transduced human B cells negatively affected Akt phosphorylation as well as some downstream targets, such as GSK-3β and PRAS40. This decrease in Akt phosphorylation was observed to be a result of Akt being physically hindered from recruiting to the plasma membrane due to significant depletion in PI(3,4)P2 levels; visualized via under confocal microscopy analysis of Akt-EGFP transfected INPP4A WT cells. Glycolysis assays determined the decrease in Akt activation may have a significantly impaired ability to increase extracellular acidification rate upon BCR stimulation in these INPP4A WT cells. In contrast, the Btk/PLCγ branch, and the subsequent calcium signaling response during BCR activation, was not affected in INPP4A WT transduced B cells. Current

results show that depletion of one of the specific products of PI3K, PI(3,4)P2, can selectively affect the activation of one of the downstream phosphoinositide-binding proteins.

Understanding the mechanisms of these pathways and how they are affected could lead to better therapeutic research against consequence of INPP4 dysregulation in B cells. Future experiments will further define the mechanisms by which PI(3,4)P2 depletion selectively affects these downstream signaling events and determine their impact on metabolic programming and B cells function *in vivo*.

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ABBREVIATIONS

2-DG – 2-Deoxy-D-glucose

4EBP1 – eIF4E-binding protein 1

70-S6K – Ribosomal S6 kinase p70

ADP – Adenosine diphosphate

AGC family – [PKA, PKG, & PKC] kinase family

Akt – Protein kinase B

AML – Acute myeloid leukemia

ANTH domain – AP180 N-terminal homology domain

ATP – Adenosine triphosphate

BAD – [B cell lymphoma 2]-associated agonist of cell death

Bam32/DAPP1 – B lymphocyte adaptor molecule of 32kDa

BCAP – B cell adaptor for PI3K

BCR – B cell antigen receptor

BLNK – B cell linker protein

BRCA1 – Breast cancer 1 gene

BSA – Bovine serum albumin

Btk – Bruton's tyrosine kinase

Ca^{2+} – Calcium

Cat. – Catalogue number

Cbl-b – Casitas B-lineage lymphoma b

CBR2/3 – C2-domain binding regions 2 & 3

CLL – Chronic lymphocyte leukemia

DAG – Diacylglycerol

ddH_2O – Double distilled water

DEPTOR – DEP[Dishevelled, Egl-10, & Pleckstrin]-domain-containing mTOR-interacting protein

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

Dox – Doxycycline

DPBS – Dulbecco's phosphate-buffered saline

E. coli – *Escherichia coli*

ECAR – Extracellular acidification rate

EEC – Endometroid endometrial cancer

EGFP – Enhanced green fluorescent protein

eIF3 – Eukaryotic initiation factor 3

ELISA – Enzyme-linked immunosorbent assay

ENTH domain – Epsin N-terminal homology domain

ER – Endoplasmic Reticulum

FBS – Fetal bovine serum

FERM domain – [4.1 protein, Ezrin, Radlxin, & Moesin] domain

FO B cells – Follicular B cells

FYVE domain – [Fab1, YOTB, Vac1, & EEA1] zinc finger domain

g – G-force

GAB1/2 – GRB2-associated binding protein 1 & 2

GC – Germinal centre

Glut1 – Glucose transporter 1

Grp1 – General receptor of phosphoinositides 1

GSK-3β – Glycogen synthase kinase-3 beta

GTP – Guanosine triphosphate

HK2 – Hexokinase 2

HPLC – High performance liquid chromatography

hr – Hour(s)

Ig – Immunoglobulin

IL-2 – Interleukin 2

IL-23 – Interleukin 23

INPP4A/B – Inositol polyphosphate 4-phosphatase type I & II

Ins – Inositides

Ins(1,3,4)P3 – Inositol (1,3,4) trisphosphate

Ins(1,3,4,5)P4 – Inositol (1,3,4,5) tetrakisphosphate

Ins(1,4)P2 – Inositol (1,4) bisphosphate

Ins(1,4,5)P3 – Inositol (1,4,5) trisphosphate

Ins(3)P – Inositol (3) monophosphate

Ins(3,4)P2 – Inositol (3,4) bisphosphate

Ins(4)P – Inositol (4) monophosphate

IP3 – Inositol triphosphate

ITAM – Immunoreceptor tyrosine-based activation motif

ITIM – Immunoreceptor tyrosine-based inhibitory motif

Lck – Lymphocyte-specific protein tyrosine kinase

Li^+ – Lithium

Lpd – Lamellipodin

LSL – Lox-STOP-Lox

Lyn – Lyk/Yes novel tyrosine kinase

M – Molar/Mole

mg – Milligram

Mg^+ – Magnesium

MHC II – Major histocompatibility complex class II

min – Minute(s)

mL – Millilitre

mLST8 – Mammalian lethal with SEC3 protein 8

mM – Millimolar

mpH – milli-pH

mTOR – Mammalian target of rapamycin

Mut – Mutant

MZ B cells – Marginal Zone B cells

nm – Nanometre

OCR – Oxygen consumption rate

PBS – Phosphate-buffered saline

PDK1 – Phosphoinositide-dependent kinase 1

PenStrep – Penicillin & Streptomycin

PH domain – Pleckstrin homology domain

PHLPP – PH-domain leucine-rich repeat protein phosphatases

PI – Phosphoinositol lipid

PI(3)P – Phosphatidylinositol (3) monophosphate

PI(3,4)P2 – Phosphatidylinositol (3,4) bisphosphate

PI(3,4,5)P3 – Phosphatidylinositol (3,4,5) trisphosphate

PI(3,5)P2 – Phosphatidylinositol (3,5) bisphosphate

PI(4)P – Phosphatidylinositol (4) monophosphate

PI(4,5)P2 – Phosphatidylinositol (4,5) bisphosphate

PI(5)P – Phosphatidylinositol (5) monophosphate

PI3K – Phosphoinositide-3 kinase

PKC – Protein kinase C

PLCγ2 – Phospholipase C gamma 2

PM – Plasma membrane

PMSF – Phenylmethyl sulfonyl fluoride (serine protease inhibitor)

pNPP – para-Nitrophenylphosphate

PP2A – Protein phosphatase 2A

PRAS40 – Proline-rich Akt substrate of 40kDa

PTEN – Phosphatase and tensin homology

PTP – Protein tyrosine phosphatase

PTPL-1 – Protein tyrosine phosphatase 1

PX domain – Phox homology domain

Raptor – Regulatory protein associated with mTOR

Rheb – Ras homolog enriched in brain

Rictor – Rapamycin insensitive companion of mTOR

rpm – Rotations per minute

RSK – Ribosomal S6 kinase

RT – Room temperature

sec – Second(s)

SGK – Serum/Glucocorticoid regulated kinase

SH1 domain – Src homology 1 domain

SH2 domain – Src homology 2 domain

SH3 domain – Src homology 3 domain

SHIP1/2 – SH2 domain containing inositol phosphate 5-phosphatase 1 & 2

SKAP – Src kinase-associated phosphoprotein

SLE – Systemic lupus erythematosus

SNX9 – Sorting nexin 9

Syk – Spleen tyrosine kinase

TAPP1/2 – Tandem PH-domain-containing protein 1 & 2

TBST – Tris-buffered saline, 0.1% Tween-20

Tet – Tetracycline

TH domain – Tec homology domain

TNF-α – Tumor necrosis factor-alpha

TSC – Tuberous sclerosis protein

WT – Wild-type

µg – Microgram

µL – Microlitre

µM – Micromolar

Chapter 1: INTRODUCTION

1.1 BCR signaling and PI3K/Akt pathways

1.1.1 B lymphocytes

1.1.1.1 What are they?

B lymphocytes, along with T lymphocytes, are subsets of leukocytes which serve as part of the host's adaptive immune response. They are partly responsible for immunological memory when a pathogen is encountered, whether naturally or through vaccination efforts [1, 2]. B cells are usually quiescent within the body. Activation of these B cells and the adaptive immune system in general, typically occurs after the innate immune response has been triggered. The innate response consists of innate cells such as macrophages, neutrophils, and dendritic cells [3].

1.1.1.2 What is their function in fighting infection?

The cornerstone of B cell function, and the overall adaptive immune system, is the generation of immune memory that is long-lived to provide a faster and more potent response to re-occurring or similar pathogens/antigens [1]. This response is mediated through the B Cell Antigen Receptor (BCR) which contains a transmembrane form of immunoglobulin (Ig) molecules that deal with the overall memory function of the B cell. These Ig's are also secreted to produce critical specific defence functions such as viral neutralization, antibody-dependent cellular cytotoxicity, complement-mediated lysis of pathogens or infectious cells, and phagocytosis [4, 5]. The Ig secreted by B cells are commonly referred to as antibodies [4]. Even though these are both Ig, the main difference between a membrane-bound Ig and an antibody is that antibodies do not contain a hydrophobic transmembrane domain to be attached to the plasma membrane (PM), whereas membrane-bound Ig's do [6, 7]. Ig's include a variety of subsets

which are distinguished by the type of heavy chain they contain: IgM, IgG, IgA, IgE and IgD [1, 4]. This diversity provides the body with different types of immune responses to deal with specific types of invading pathogens, different stages of disease progression, or changing of hands during defence efforts [2, 5, 8].

1.1.1.3 Where and how are they developed and activated?

All immune cells are descended from haematopoietic stem cells [9]. Immature B cells are generated in the bone marrow from lymphoid precursors. During this development, a unique recombination process takes place involving the variable region of the Ig heavy and light chains which are responsible for antigen recognition, binding, and activation of the B cell [1, 6]. This variable region consists of V, (D), and J gene segments and together, they give the B cells their unique BCR to bind to and be activated by specific or closely related foreign pathogens [10-12]. Whilst still in the bone marrow, the immature B cells undergo positive and negative selection, before becoming mature naïve B cells to be released to circulate within the body. These tightly regulated selection processes ensure that the mature B cell population only express functional BCR's that are non-autoreactive, which could be detrimental [1, 2, 10].

When mature B cells are activated via the BCR, they proliferate and differentiate within secondary lymphoid organs (i.e. the spleen and lymph nodes) in a T cell-independent or -dependent manner. B cells that differentiate and proliferate in a T cell-independent manner include B1 cells which are an important source of circulating low-affinity predominately IgM antibodies, or Marginal Zone (MZ) B cells which are non-circulating, rapid-responding B cells located in the splenic marginal zones [10, 12]. Alternatively, Follicular (FO) B cells can be activated via the BCR and by presenting internalized antigens in an MHC II- and T cell-dependent manner to become the long-lived "conventional" memory B cells that are observed in

humoral immune responses. Activated FO B cells proliferate and differentiate within germinal centre (GC) areas of lymphoid tissues. Long-lived Plasma cells also arise from T cell-dependent interactions which maintain antigen-specific antibodies within the blood. Pre-formed antibodies can generate immune complexes when the specific antigen is encountered again which in turn assists in generating more rapid memory responses [8, 11].

1.1.2 B cell antigen receptor

1.1.2.1 Immunological role of BCRs

The most important role of the BCR is to ensure highly specific responses to all foreign antigens the host encounters on a day-to-day basis. It does this by having a high degree of Ig diversity within the B cell population [1, 6]. The current theory suggests during development and maturation, immature B cells are equipped with differing BCRs that bind distinct antigens based on their unique variable regions. Simultaneously, negative and positive selection remove those cells that react to self-antigens or do not exceed the affinity threshold to any antigen via programmed cell death [13]. After maturation to naïve B cells, whenever one of these naïve B cells binds to a specific foreign antigen, that B cell is then activated and undergoes rapid proliferation to produce a colony of B cells that specifically target that particular threat [14].

BCR's are comprised of two parts – the main membrane-bound Ig receptor and the signal transduction components [15]. The Ig receptor is a heterodimer that antigens bind to and are comprised of 4 polypeptides, two heavy chains and 2 light chains. The heavy chains determine the isotype of Ig (e.g. IgD, IgM, IgA, IgG, or IgE), whereas the light chains, also known as the variable chains, are either kappa or lambda isotypes [6, 16]. During assembly in the endoplasmic reticulum (ER), the paired heavy and light chains becomes non-covalently associated with the transmembrane signal transduction heterodimer CD79 and the BCR complex is transported to the

cell surface. Once a specific antigen has bound to the BCR it can generate a signal to activate the downstream signaling pathways in B cells [6, 11, 15].

1.1.2.2 How do these receptors activate the B cell?

Much like the CD3 chains of T cell receptors, CD79 contains cytoplasmic tails (Ig-α/Ig-β) with immunoreceptor tyrosine-based activation motifs (ITAMs) which are crucial for signaling transduction during receptor ligation [11, 17, 18]. BCR ligation activates protein tyrosine kinases such as Lyn, Syk, and Btk, phosphorylating Ig-α/Ig-β at the conserved tyrosine-containing motifs ($YxxL/I_{(6-8)}YxxL/I$). This phosphorylation provides a docking site for proteins that contain the phosphotyrosine-binding SH2 domains to continue the activation of downstream signaling proteins for cell activation, metabolism, and survival (**Fig. 1A**) [17, 18].

The balance of initiation, amplitude and duration of BCR activation can be influenced by activating and inhibitory signaling proteins (**Fig. 1**). The expression of adaptor proteins GAB1/2, BLNK, BCAP and CD19 serve as docking sites for recruitment of initial downstream signaling proteins to amplify BCR activation signals [15]. However, BCR signaling must also be silenced to prevent prolonged activation when continuously exposed to foreign and autoantigens. The tyrosine kinase Lyn has a dual role in BCR activation and inhibition as it phosphorylates the tyrosine residues in the ITAM-containing receptors to activate cells, but can also counteract this activation by phosphorylating the single tyrosine residue within immunoreceptor tyrosine-based inhibitory motif (ITIM) domains on CD22 and FcγRIIβ [18, 19]. Phosphorylation of ITIM containing receptors provides a docking site for downstream phosphatases SHP1 and SHIP1 to negatively regulate signaling pathways and inhibit downstream signals for cell activation, proliferation, and

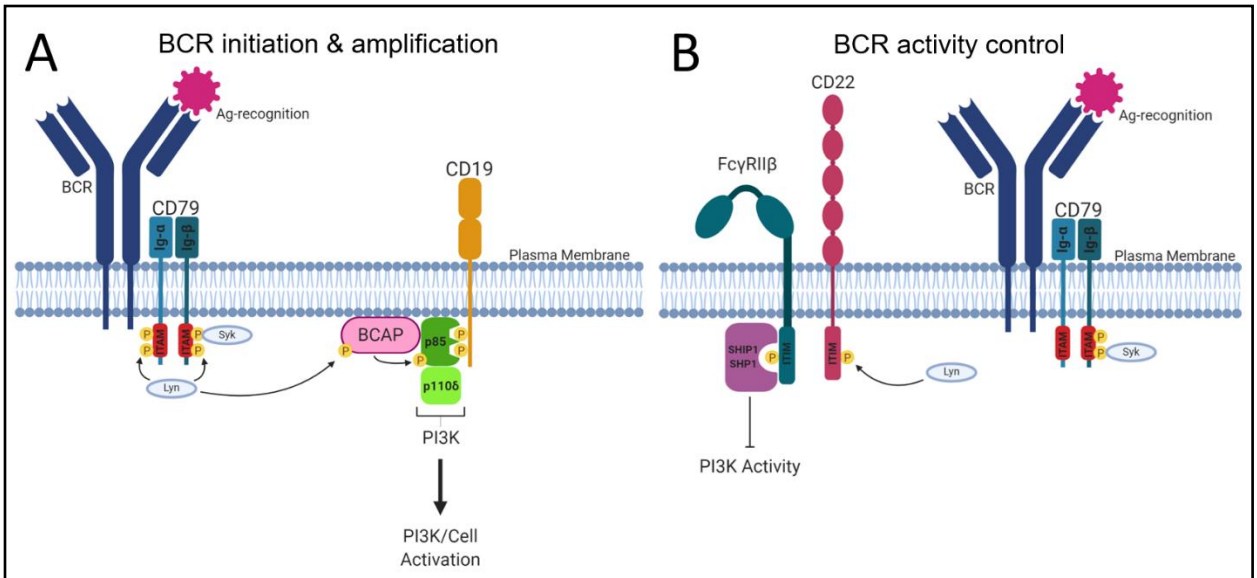


Figure 1. <u>Initiation, amplification, and activity control of BCR activation.</u>

The balance of initiation, amplitude, and duration of BCR activation is influenced by activating and inhibitory signaling proteins. **(A)** Foreign antigens bind to the variable light chains on the membrane-bound Ig of the BCR. The signal transfers to the CD79 signal transduction receptor associated with the BCR. ITAM domains on the CD79 are phosphorylated by Lyn which then phosphorylate the co-receptor CD19 and adaptor protein BCAP needed for BCR amplification. Activated BCAP interacts with the p85 subunit of PI3K, recruiting it to the PM and binding onto CD19, allowing for p110 catalytic subunit activity to occur. This commences further B cell activation by activating downstream signaling targets. **(B)** Duration of BCR activation is controlled through the phosphorylation of ITIM domains on inhibitory co-receptors CD22 and FcγRIIβ by Lyn, allowing for docking sites for proteins inhibiting PI3K/BCR activation.

survival (**Fig. 1B**) [15, 18, 19]. To summarize, the activation, maintenance, and shut down of BCR activation is complex due to the tight regulations and checkpoints ensuring proper B cell function and it is all done through the balance between numerous kinases and phosphatases.

1.1.3 PI3K signaling pathway

The Phosphoinositide-3-kinase (PI3K) signaling pathway is critical in regulating the cell cycle and is linked to cell quiescence, proliferation, and survival. However, when this pathway is dysregulated, it leads to various consequences such as cancer, autoimmune disease, and abnormal lymphocyte development and function [20]. Therefore, this conserved pathway's activity is tightly regulated by various kinases and phosphatases in a strict multistep progression [21].

1.1.3.1 How it is activated during BCR ligation

PI3K belongs to a heterodimeric family of lipid kinases activated by various cell surface receptors including such as growth receptors, co-stimulatory receptors, cytokine and chemokine receptors, toll-like receptors, and in the context of B cells, antigen receptors [22]. PI3K is expressed in three different classes, Class I, Class II, and Class III [23]. The Class IA PI3K contains the catalytic p110 subunit and the regulatory p85α (most common), p85β, or p55γ subunit [22, 24]. There are four isoforms of Class I PI3K containing distinct p110 subunits p110α, p110β, p110γ, and p110δ. The p110α and p110β isoforms are ubiquitously expressed, whereas p110γ and p110δ are mainly restricted to functions in immune cells, including B cells [25]. The engagement of BCR initiates the activation of PI3K via the co-receptor CD19 and the cytosolic adaptor protein BCAP (**Fig. 1A**) [26]. BCR-associated protein B cell adaptor for phosphoinositide3-kinase (BCAP) is key for BCR signaling amplification [27]. BCAP is required for PI3K recruitment and activation at the plasma membrane (PM). BCAP accumulates

to the PM after PI3K binds to the tyrosine phosphorylated YxxM sequence of BCAP through its p85 subunit [23]. The recruitment of PI3K to the PM by BCAP allows the function of the catalytic p110 subunit to commence. Similarly, the transmembrane protein CD19 contains a YxxM motif that can recruit and activate PI3K. BCAP or CD19 deficiencies in B cells have been shown to affect the production of B1 cells, and the immature to mature development of conventional B cells, leading to attenuated humoral responses [28].

Once recruited and activated, Class I PI3Ks phosphorylate the second messenger phosphoinositol (PI) lipid phosphatidylinositol (4,5) bisphosphate (PI(4,5)P3) at the D-3 position to produce PI(3,4,5)P3 [24]. All classes of PI3K phosphorylates at the D-3 position and can produce PI(3)P and Class I and II in producing PI(3,4)P2 *in vitro*. However, only Class I PI3K can produce PI(3,4,5)P3 from PI(4,5)P2 (**Fig. 2**) [25]. Downstream signaling pathways required for cell activation are triggered via binding to PI(3,4,5)P3 and PI(3,4)P2, including the two major effector signaling proteins, Btk and Akt [26]. Levels of PI(3,4,5)P3 are controlled by further hydrolysis into PI(3,4)P2 and PI(3)P by various phosphatases or reverted back to PI(4,5)P2. These phosphatases, which will be described in greater detail below, negatively regulate the PI3K pathway and the overall activation of B cells by depleting PI lipids thus preventing detrimental hyperactivation [22, 23, 26].

Impairments in B cell development, peripheral subset differentiation, and function have been shown to be a consequence of defects in the regulatory p85α or catalytic p110δ subunits of PI3K [29]. Mutations in p85α gives rise to various cancers such as endometroid endometrial cancer (EEC), non-EEC, breast, ovarian, and colon cancers. This particular mutation disrupts the interactions between the p85α and p110 domain prior to proper cell activation. This disruption

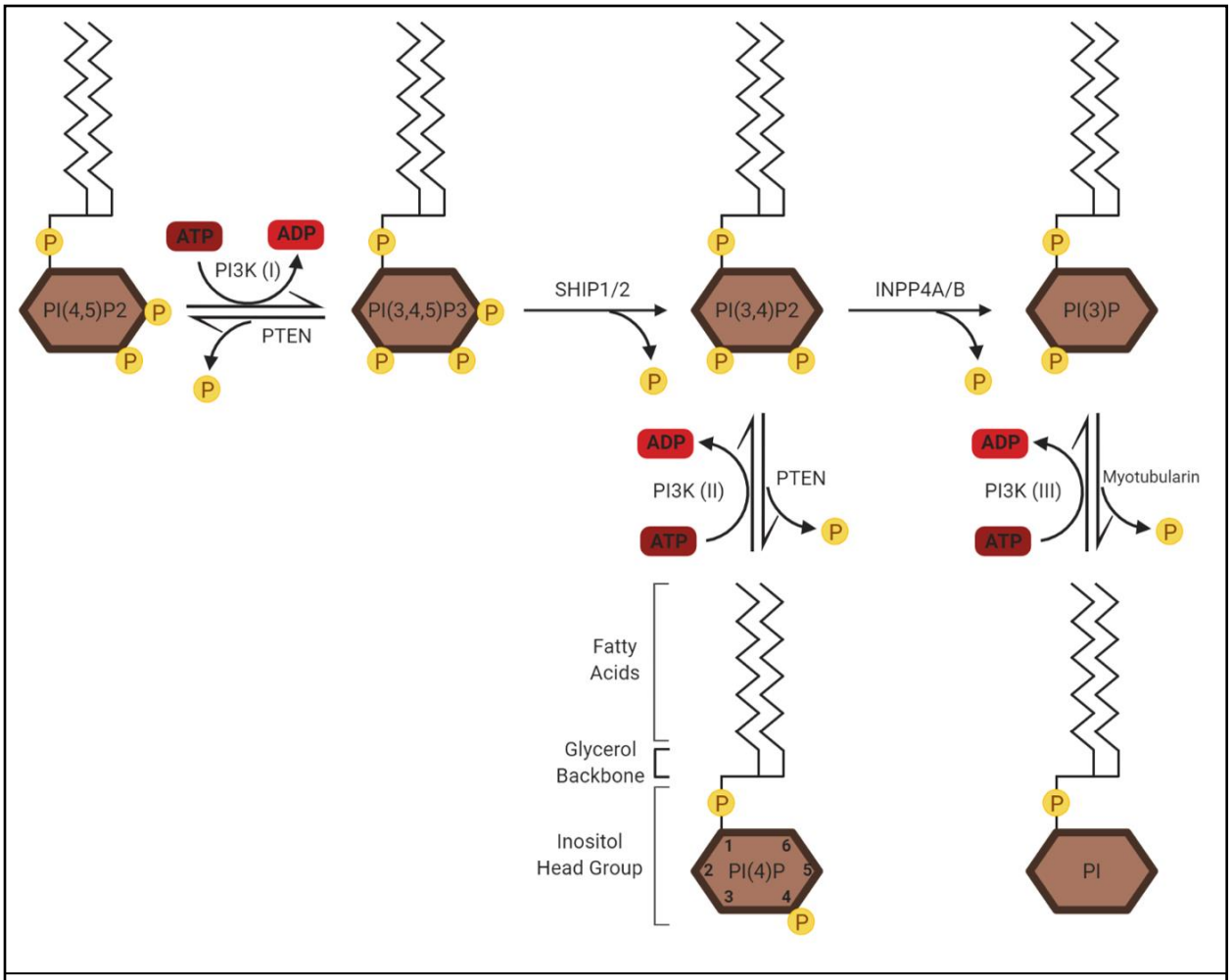


Figure 2. <u>Phosphatidylinositol phosphates/phosphoinositides production and regulation.</u>

Phosphoinositol (PIs) lipids are comprised of fatty acid chains which bury in the plasma membrane and an inositol head group connected by a glycerol backbone. Kinases and phosphatases add and remove phosphates from hydroxyl groups, respectively, to the inositol head group at positions D-3, D-4, and D-5. Different phosphorylated combinations provide docking sites for different PH-domain containing signaling proteins involved in cell activation, metabolism, proliferation, and survival. PI(3,4,5)P3 and PI(3,4)P2 are produced by the Class I PI3K pathway and are major branching points for Btk/PLCγ and Akt activation and downstream signaling pathways.

releases the inhibition of p110 thus promoting downstream signaling activity and cell hyperactivation [24]. p110δ mutations can cause a form of immune deficiency stemming from excessive PI3K activation and the hyperactivation of Akt which is essential in cell growth, proliferation, and survival. This leads to the blockage of B cell development with increased transitional cells, fewer circulating memory cells, and immunodeficiency symptoms [22]. Typically, treatments for PI3K hyperactivation involves targeting downstream targets that are subsequently overactivated like rapamycin for mTOR [24]. Phase I trials of pan-PI3K inhibitors (ex. GDC-0941 BKM120, PX866, BAY 80-6946 and XL-147 for Class I PI3K) are underway for patients diagnosed with various cancers. However, isoform specific inhibitors like Cal101 (Idelalisib) against the p110δ subunit are an approved treatment for patients with chronic lymphocyte leukemia (CLL), a cancer resulting in the disproportionate proliferation of B cells [15, 24, 30].

1.1.3.2 Overview of PI3K-dependant pathways – PI binding proteins

The recruitment and binding of signaling proteins to the PI's during PI3K activation is made possible by their Pleckstrin Homology (PH) domains [31]. Various studies, through membrane recruitment assays and *in vitro* biochemical analyses, looked at the binding between PI's and proteins containing PH-domains. Approximately 10% of all PH-domain proteins have the ability to bind to PIs with high affinity, and there are many PH-domain containing proteins expressed within B cells [31]. Proteins found to specifically bind to PI3K products PI(3,4,5)P3 and/or PI(3,4)P2 include Grp1, Btk, PLCγ2, PDK1, SKAP, Akt, Bam32/DAPP1, and TAPP1/2 [20, 32]. These PH-domain containing proteins have high affinity binding to PIs with a pair of adjacent phosphates situated on their respective headgroups [31].

Two branches of the PI3K pathway required for cell activation are initiated by the two major effector signaling proteins, Btk and Akt (**Fig. 3**) [26]. Btk is a protein tyrosine kinase that mediates B cell activation via the activation of PLCγ2, which hydrolyzes PI(4,5)P2 and ultimately leads to release of internal calcium held in the endoplasmic reticulum (ER) [33]. Akt is a critical serine-threonine kinase in the PI3K pathway that mediates cell growth, metabolism and survival through its role in activating a number of downstream targets [34]. The requirement of these PI's in the activation of the Btk and Akt branches of PI3K signaling emphasizes important roles of PI's in the regulation of protein kinases as well as the importance of phosphatases regulating PI levels.

1.1.4 Phosphoinositides and their phosphatases

1.1.4.1 What are PIs?

Phosphoinositol (PI) comprises a minor component of cell membrane lipids in eukaryotes [32]. The inositol headgroup of PI can be phosphorylated on three positions to produce the phosphoinositides PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3,4,5)P3 [35, 36]. The phosphates on these lipids make them an acidic phospholipid and their function is a simple one – to interact with proteins. These proteins include protein kinases, phospholipases, regulators of membrane trafficking, cytoskeletal proteins, scaffold proteins, and ion channel proteins [37]. Therefore, phosphorylated PI species are associated with different aspects of cell function and activation including cell metabolism, proliferation, calcium influx and vesicular sorting. Alterations in PI levels lead to cancer and other diseases [37, 38]. The PI3K pathway generates three types of PI's: PI(3,4,5)P3, PI(3,4)P2, and PI(3)P (**Fig. 3**). PI(3)P is mainly generated by class III PI3K which is not activated by BCR signaling (**Fig. 2**). For each PI, there

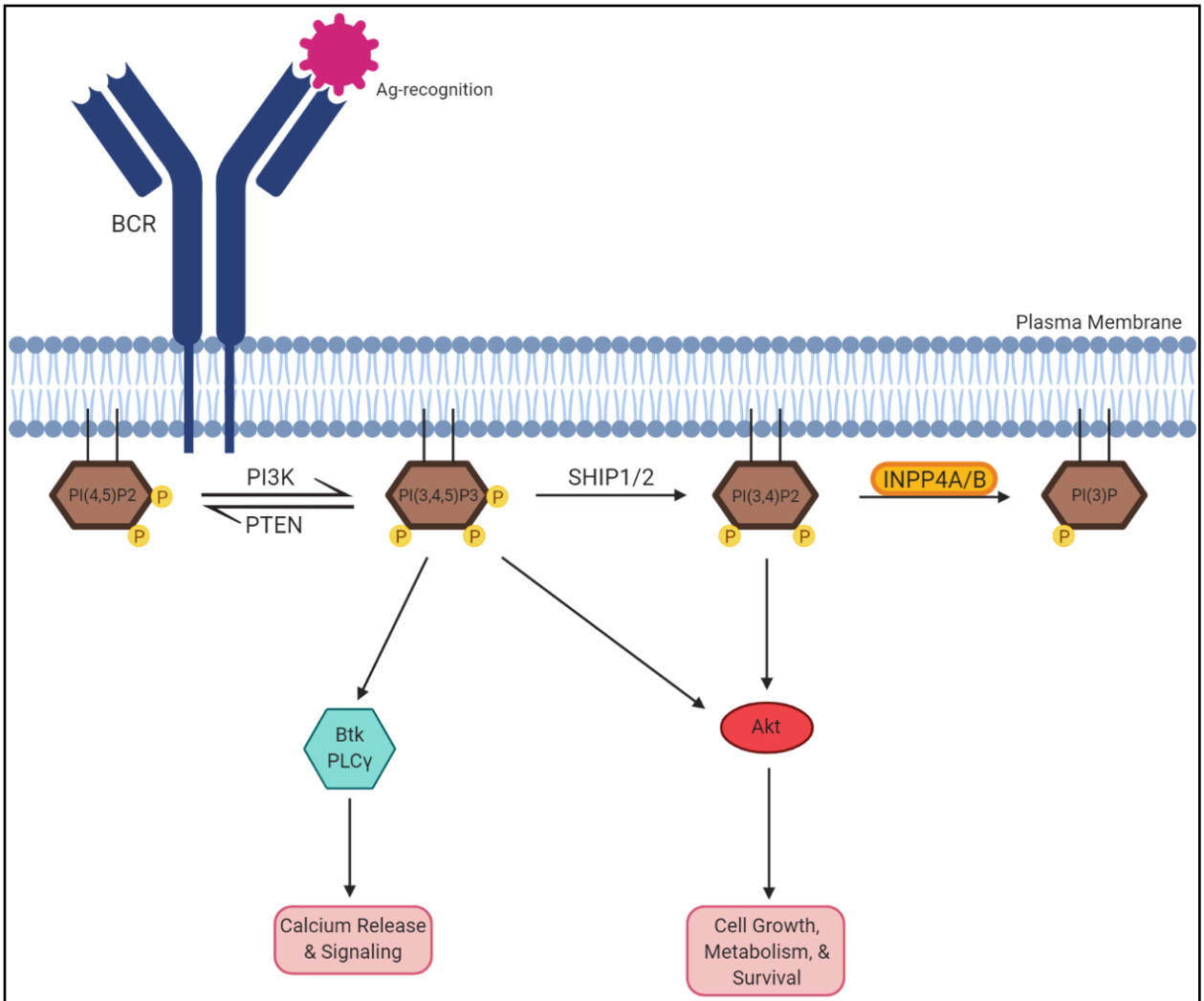


Figure 3. <u>Simplified overview of PI3K activation and the two major signaling branches.</u>

PI3K function is activated during BCR ligation, producing PI(3,4,5)P3 from PI(4,5)P2. PTEN, SHIP and INPP4 are negative regulators the PI3K pathway through the hydrolysis of their respective PI's. PI(3,4,5)P3 is hydrolysed by SHIP1/2 and PTEN to PI(3,4)P2 and PI(4,5)P2, respectively. PI(3,4)P2 is hydrolysed to PI(3)P by INPP4A/B (phosphatase of interest). The two major signaling branches of the PI3K pathway required for further cellular activation during BCR stimulation are the Btk/PLCγ and Akt branches. The Btk/PLCγ branch is vital for calcium release and signaling while Akt is pivotal for promoting cellular growth and metabolism while inhibiting apoptotic factors thus promoting survivability.

are phosphatases that alter the levels present in the cell during cell stimulation and are involved in their negative regulation [38].

The PI3K pathway progresses mostly in a forward motion once BCR ligation occurs in order to activate the downstream pathways for cell activation, proliferation, and survival. As discussed above, during BCR binding the PI3K becomes activated and phosphorylates PI(4,5)P2 to PI(3,4,5)P3. Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 and 2 (SHIP1/2) can then hydrolyse PI(3,4,5)P3 to PI(3,4)P2 which is further hydrolysed to PI(3)P by inositol polyphosphate 4-phosphatase (INPP4) [39-41]. Another phosphatase that regulates the PI3K pathway is the phosphatase and tensin homolog (PTEN) that can hydrolyse PI(3,4,5)P3 back to PI(4,5)P2 or hydrolyze PI(3,4)P2 to PI(4)P (**Fig. 2 and 3**) [39, 42]. These three phosphatases regulate the overall amplitude and duration of the PI3K signaling pathway by controlling the PI levels. There is further evidence that phosphatases can differentially control PI levels in specific regions of the cell membrane, relevant in cell polarity and migration. This spatio-temporal regulation of PI pools ultimately determines the binding ability and activation of downstream binding targets of PI(3,4,5)P3 and PI(3,4)P2 to maintain B cell signaling homeostasis and prevent hyperactivation [39]. These downstream targets include the PH-domain containing proteins such as Akt, Btk, TAPP2, and their downstream targets including PLCγ, GSK-3β, mTOR, PRAS40, and p70-S6K (**Fig. 4 and 5**). These proteins and their functions in the context of PI3K pathway and B cell activation will be described in greater detail below.

1.1.4.2 PTEN & SHIP

PTEN is ubiquitously expressed while SHIP1 is strictly a hematopoietic cell protein [43]. PTEN and SHIP are 3- and 5-phosphatases, respectively, that negatively regulate in different ways the PI3K-mediated cell growth, metabolism, and survival in hematopoietic cells such as T

and B cells [43]. While they both control the levels of PI(3,4,5)P3 within cells, they dephosphorylate the D-3 and D-5 phosphate to respectively produce distinct products PI(4,5)P2 or PI(3,4)P2. Both PTEN and SHIP can decrease Akt and Btk binding and activation of downstream signaling targets [40]. Individually, PTEN is considered as the limiter of the pro-growth activity during cellular activation as it determines the levels of PI(3,4,5)P3 available for the subsequent steps of the PI3K pathway. Whereas SHIP moves the PI3K pathway forward and produces docking sites for PI(3,4)P2-specific binding proteins, thus promoting activation of distinct aspects cellular growth and survival [36].

With PI3K activation needing to be tightly regulated through these phosphatases, their dysregulation does lead to severe consequences. In T cells, deficiency in PTEN results in lethal T cell lymphomas [43]. In B cells, deficiencies in PTEN and SHIP1 can lead to cancer and autoimmune diseases [20]. During B cell and BCR development, approximately 70% of these BCRs will bind and react to self-antigens. Therefore, to prevent consequential autoimmunity, B cells with low level autoreactivity which are not negatively selected need to experience anergy [44]. SHIP is an important regulator of B cell anergy in order to maintain unresponsiveness in the mature B cell pool. An acute loss of SHIP will lead to an increase in self-antigen responsiveness and complete knockout in mice saw an increase in autoantibody production and diseases similar to Systemic Lupus Erythematosus (SLE) [40, 44]. B cell regulation by SHIP has been found to occur by its recruitment to phosphorylated ITIM and partially-phosphorylated ITAM regions of CD22 and CD79α/β receptors, respectively. In addition to hydrolysing PI(3,4,5)P3, SHIP may regulate B cells by increasing PI(3,4)P2 to recruit TAPP1/2 which inhibit B cell activation [40, 41, 44]. SHIP deficient B cell studies observed $SHIP^{-/-}$ knockout mice had a reduction in B cell compartments but increased BCR-induced proliferation *in vitro* [43]. As

well, through the ITIM inhibitory receptor interaction, SHIP promotes negative selection escape from the induction of high levels of pre-BCR signaling in pre-B leukemia cells thus promoting cell survival [40]. In CLL patients, both SHIP expression and the phosphorylation at Y1022 were decreased in the more aggressive cases [40].

PTEN was first identified as a tumor suppressor as it was commonly observed to be decreased in expression or completely lost in many human cancers, as it ultimately regulated the cell from over activation and proliferation [45]. It is also commonly lost with other phosphatases such as INPP4B in 50% of human breast cancer patients, thus leading to reduced survival outcome [41]. More so, germline mutations of PTEN within individuals puts them at a predisposed risk to developing a range of cancers within their lives [41]. In B cell-specific PTEN knockout mice, MZ or B1 cell differentiation was favoured and these cells exhibited hyperresponsiveness to *in vitro* stimulation due to excessive PI(3,4,5)P3 levels [43]. SHIP or PTEN specific knockouts in B cells are individually insufficient for tumorigenesis in mice and are infrequently seen in human B cell malignancies [40, 43]. However, when PTEN is co-deleted with SHIP1, it leads to the development of spontaneous and lethal B cell lymphomas due to the increase to PI(3,4,5)P3 levels allowing for the increase in Akt phosphorylation [20, 41, 43, 46].

With the extensive studies on the consequences of PTEN and SHIP deficiencies, various small molecules drugs have since been developed to target the activity of these enzymes. For autoimmune disease, small molecule drugs like AQX-1125 promotes SHIP phosphatase activity by attaching to the C2 domain and are in anti-inflammatory clinical trails for symptoms like mild-moderate asthma and bladder inflammatory disease. Curiously, other SHIP-promoting compounds have also been demonstrated to help reduce the viability of multiple myeloma cell

lines and inhibitors have been demonstrated to promote apoptosis, possibly because of a balance of both PI(3,4,5)P3 and PI(3,4)P2 for survival and to avoid activation-induced cell death [40]. On the other hand, compounds like 3AC act as inhibitors are immunomodulatory and even anti-inflammatory by possibly preventing the hydrolysis of soluble Ins(1,3,4,5)P4 by SHIP [40]. With PTEN ubiquitously expressed in the body, and having both phospho-tyrosine phosphatase and PI phosphatase activity, reversible and relatively specific small molecule PTEN-inhibiting drugs have been developed to alleviate many symptoms of diseases caused by PTEN dysregulation [47]. PTEN inhibition drug studies and clinical trials, for example with Vanadium and Peroxovanadium, indicate potential uses in cell regeneration processes (neurodegenerative conditions like Alzheimer's disease, tissue injury, or ischemia, and even insulin resistance metabolic disorders), infertility, stem cells-based therapy (maintenance and self-renewal), cancer-related diseases in certain circumstances, insulin-resistance metabolic diseases especially Type 2 Diabetes, and even pain relief, by increasing Akt binding, activation, and cell growth [42]. However, a deeper investigation is still needed as they have a potential to influence tumorigenesis [42].

1.1.4.3 INPP4

Inositol polyphosphate 4-phosphatase (INPP4) is a Mg^{+}-independent phosphatase approximately 105-110kDa in size [38, 48-50]. It has two main isoforms, INPP4A and INPP4B, transcribed from the INPP4 type I and II genes, respectively and share 37% sequence homology to each other [38, 41, 51]. INPP4 is ubiquitously expressed in the body with various expression of the two isoforms within individual tissues [41]. The primary function of INPP4 is negatively regulating the PI3K signaling pathway by hydrolyzing the D-4 phosphate specifically from PI(3,4)P2, reducing its levels during cell activation [20, 41].

Over the last approximately 30 years, INPP4's function has been studied in various cell lines and physiological consequences observed when this phosphatase is dysregulated. Particularly, its been found to have dual oncogenic driving and suppressive as well as chemoresistance roles in various cancers including breast cancer, acute myeloid leukemia, and epithelial cancer [52-55]. INPP4 has also been implicated in neural regulation, innate immunity activity, bone maintenance, and airway inflammation control [20, 41, 46, 56, 57]. However, its role in BCR activation and B cell metabolism has not been studied. A more comprehensive review of INPP4 and its function will be provided below.

1.1.5 Phosphoinositide-dependant signaling pathways

The 3-phosphoinositides, PI(3,4,5)P3 and PI(3,4)P2, act as a docking site for various adaptor proteins with domains like PH (the most common), PX, FYVE, ANTH, ENTH, and FERM [58]. They act as secondary messengers and mediate B cell activation after BCR ligation by acting as docking sites for signaling proteins and initiate PI(3,4,5)P3- and PI(3,4)P2-dependent branches of the PI3K signaling pathway.

1.1.5.1 PI(3,4,5)P3-dependant signaling pathways

PI(3,4,5)P3 is a critical secondary molecule that impacts many cellular functions, including cell proliferation, calcium influx, cytoskeletal organization, and vesical-mediated transport like exocytosis, phagocytosis, and pinocytosis [32, 41]. In B cells, PI(3,4,5)P3 levels rapidly increase and max within 30sec of BCR-ligation and then quickly return to lower levels [20, 37, 59]. PI(3,4,5)P3 is an essential docking site for various PH-domain containing proteins including Btk, PLCγ2, PDK1, Grp1, Akt, SKAP and Bam32/DAPP1. Among these, some are highly specific binders of PI(3,4,5)P3, such as Btk, while others bind similarly well to either PI(3,4,5)P3 or PI(3,4)P2, such as Akt. Thus, I will focus my discussion on downstream

signaling via Btk, due to its high specificity for PI(3,4,5)P3 binding and its functional importance in B cells.

The non-receptor tyrosine kinase Bruton's tyrosine kinase (Btk) and the phospholipase PLCγ2 both selectively bind to PI(3,4,5)P3 produced by the PI3K pathway during BCR-ligation (**Fig. 4**) [58, 60, 61]. Btk is a member of the Tec family of kinases [61]. Other nonreceptor tyrosine kinases are also activated during BCR stimulation including Src and Syk. Btk has only been shown to be expressed in B cells and in myeloid cells [33]. Btk is an important mediator of B cell development, differentiation, function, and survival as it regulates a major aspect of the PI3K pathway including the protein kinase C (PKC) and the PLCγ2 signaling pathways [61, 62]. It has also been suggested Btk also plays a role in cytoskeletal reorganization and motility [33]. Btk is vital for the release of calcium from the ER during BCR-stimulation and does so by phosphorylating critical tyrosines in PLCγ2 [33, 61].

During BCR-ligation and PI3K activation, Btk is tyrosine-phosphorylated at Y551 by another tyrosine kinase Syk and then in turn phosphorylates PLCγ2 on the four critical tyrosines residues Y753, 759, 1197, and 1217 [63]. It has been depicted that Casitas B-lineage Lymphoma b (Cbl-b) and B cell linker protein (BLNK) are needed to recruit and associate PLCγ2 to active Btk and Syk and to form an active complex for calcium release [17, 63]. Phosphorylated PLCγ2 hydrolyzes PI(4,5)P2 to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 actuates the release of calcium from the ER via binding to IP3 receptors (**Fig. 4**) [61, 63]. This release of calcium is pivotal for the further activation of downstream signaling targets that regulate immune

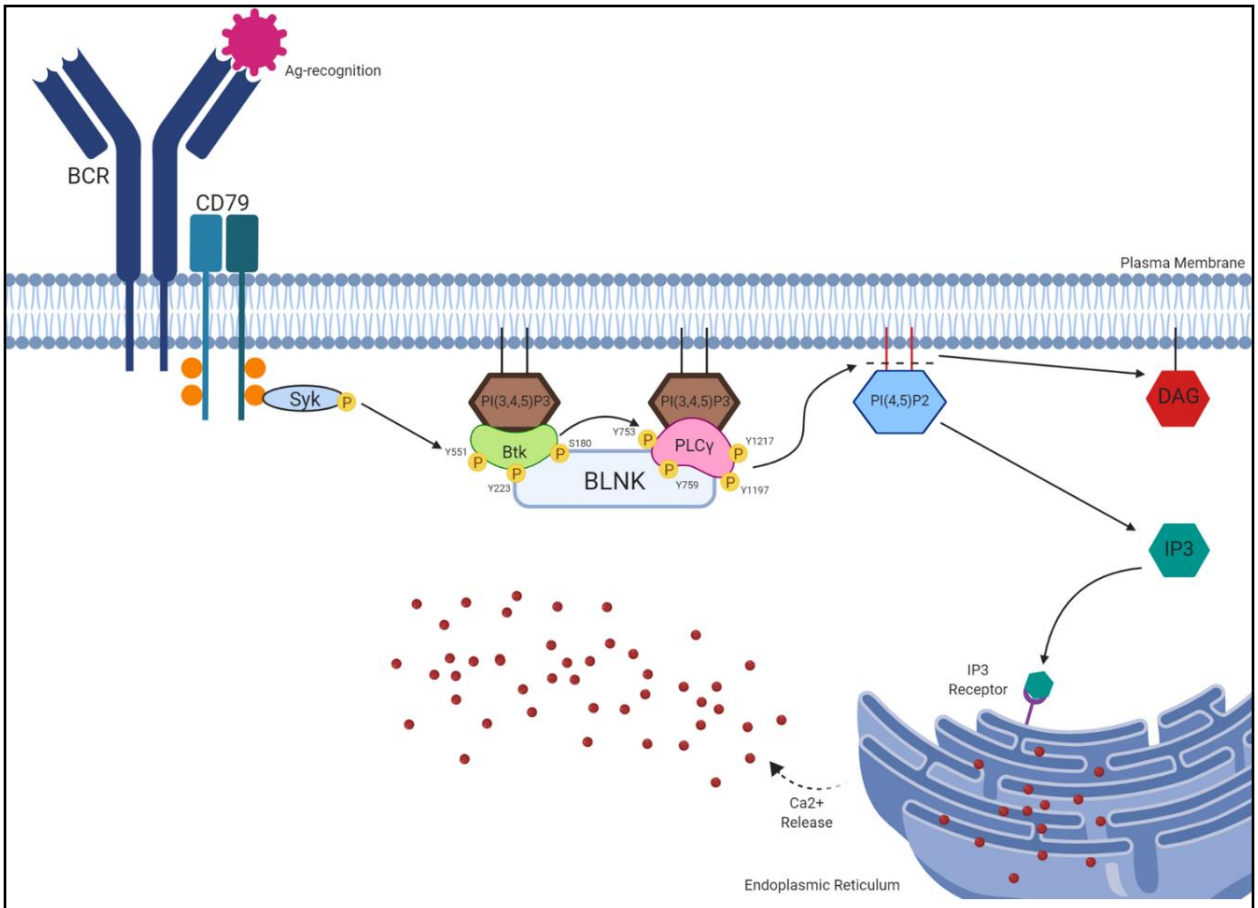


Figure 4. <u>PI(3,4,5)P3 and downstream targets – The Btk/PLCγ branch.</u>

PI(3,4,5)P3 is produced by the phosphorylation of PI(4,5)P2 by PI3K during BCR stimulation. Btk and PLCγ form a complex with BLNK to be recruited to the PM. Btk strictly binds to PI(3,4,5)P3 by its PH-domain. Btk regulates PLCγ signaling pathway and is vital for the release of calcium from the ER during BCR-stimulation. Btk becomes fully activated when phosphorylated at Y223, Y551 and S180 by the Lyn/Syk tyrosine kinases [33, 61]. Btk phosphorylates PLCγ at tyrosine residues 753, 759, 1197, and 1217. Phosphorylated PLCγ hydrolyzes PI(4,5)P2 to IP3 and DAG. IP3 binds onto IP3 receptors on the surface of the ER, triggering the release of internal calcium.

cell activation [61]. The DAG on the other hand activates PKC, consecutively prompting numerous other downstream responses including, cell proliferation, gene expression, protein secretion, and inflammatory responses [60, 64].

Btk deficiencies in B cells lead to a significant decrease in calcium response post-BCR stimulation, and negative effects on B cell development and function. This usually is characterized by little to no cell proliferation and maturation, as well sensitivity to apoptosis as Btk also has a protective role against Fas-mediated apoptosis [33, 61]. This downregulation of B cell proliferation and maturation leads to decreased levels of secreted antibodies, and an increased susceptibility to infections; a condition known as human X-linked agammaglobulinemia [65]. If there is a mutation in one of the four critical tyrosine residues in PLCγ2 there is a reduction in calcium release, due to decrease IP3 production. Mutations in all four residues results in complete elimination of Btk-dependent PLCγ2 phosphorylation during BCR contact [63]. Recently, drugs targeting Btk have been developed and used clinically to treat B cell malignancies. CLL patients treated with the drug Ibrutinib show clinical antitumor activity, and a favourable therapeutic index in these patients [65, 66]. Ibrutinib specifically targets B cell by covalently binding to Btk on C481, inhibiting Btk's ability to mediate interactions with the tumor microenvironment and decrease CLL proliferation and survival [65].

1.1.5.2 PI(3,4)P2-dependant signaling pathways

PI(3,4)P2 is the product of the hydrolysis of PI(3,4,5)P3 by the 5-phopshatase SHIP when PI3K is activated (**Fig. 3**) [36]. PI(3,4)P2 can also be produced by the direct phosphorylation of PI(4)P by Class II PI3Ks (**Fig. 2**), however this pathways of synthesis does not seem be a significant contributor to PI(3,4)P2 levels in cells activated via receptors that trigger Class I PI3Ks [67]. During cell activation, the levels of PI(3,4,5)P3 and PI(3,4)P2 increase and decrease

at different rates on the PM. PI(3,4,5)P3 rapidly increase and then return to basal levels within a span of 60sec during BCR stimulation [20, 37, 59]. In contrast, PI(3,4)P2 levels will peak at approximately 60sec, remain at plateau levels for prolonged periods, and then slowly decrease in the span of 30min [20, 36, 59].

The regulation of PI(3,4)P2 within cells is controlled by both PTEN and INPP4 (**Fig. 2**) [36, 67]. PTEN has been reported to provide roughly 57% of total PI(3,4)P2-phosphatase activity in MCF10 cells, with INPP4A and INPP4B providing the rest [67]. This may explain why deficiency in INPP4A or INPP4B alone typically is not sufficient to induce spontaneous cellular transformations unless it was co-deficient with PTEN [36, 68]. In regard to PI3K signaling, PI(3,4)P2 has typically been considered as the by-product of the negative regulation of PI(3,4,5)P3 from SHIP activity, however the existence of three phosphatases controlling its accumulation (PTEN, INPP4A and INPP4B) suggest it has unique functional importance. With new analytical techniques, it is now evident that PI(3,4)P2 is not just a by-product of PI(3,4,5)P3 removal, but in fact a second critical messenger molecule in cell growth, metabolism, survival, and membrane trafficking, and cellular metastasis [36, 58, 67, 68].

Signaling proteins containing PH-domains bind to both PI(3,4)P2 and PI(3,4,5)P3 with ranging binding affinity between the two PI lipids such as Akt, SKAP, Bam32/Dapp1, or strictly to PI(3,4)P2 such as lamellipodin (Lpd) and TAPP1/2 [20, 36, 68, 69]. This specific binding of TAPP1/2 has made it the preferable probe to observe PI(3,4)P2 accumulation and degradation [68]. TAPP1/2 can be modified to bind to PI(3,4,5)P3 by introducing a point mutation within its PI-binding pocket to alleviate the steric hinderance with the D-5 phosphate of PI(3,4,5)P3 [58]. It was suggested TAPP1/2 contributes in a feedback loop to negatively regulate the PI3K downstream targets as it recruits the tumor suppressor protein tyrosine phosphatases (PTPL-1) to

the PM, inhibiting Akt activity [36, 58]. Excessive accumulation of PI(3,4)P2 due to failure to regulate PI3K activation, most commonly from dysregulation of the phosphatases in this pathway, affects the activation of it downstream targets, usually leading to cancerous transformation and pro-growth [36].

In stimulated B cells, another PI(3,4)P2-specific binding protein Lpd was found to colocalize to accumulated pools of PI(3,4)P2 and play a role in B cell migration [69, 70]. Lpd is considered a connection point between upstream signaling pathways during the initial steps of cell activation and cytoskeletal remodeling within cells which is pivotal in physiological processes for normal immune cell trafficking. It regulates cytoskeletal dynamics including lamellipodial protrusions critical in actin dynamics (promotes actin polymerization), cell polarity, and motility, and adhesion [69, 70]. B cell motility and directionality of migration has shown to be PI(3,4)P2-dependent as depletion of PI(3,4)P2 levels via overactivity of the PI(3,4)P2 phosphatase INPP4 displayed an overall reduction of malignant B cell motility and directionality [69].

1.1.6 Akt and downstream signaling

1.1.6.1 Structure and regulation of Akt

Akt has been regarded as a pivotal step in cellular activation as it regulates downstream signaling targets for cell proliferation, metabolism, and anti-apoptotic factors [29, 34]. It is a part of the AGC kinase family and has three isoforms, Akt1, Akt2, and Akt3. These isoforms all share an N-terminal PH-binding domain and a C-terminal regulatory serine and threonine kinase domain [71]. Akt1 is the most commonly expressed isoform as it is ubiquitously expressed, whereas Akt2 and Akt3 isoforms are highly expressed in insulin-responsive tissues and the brain, respectively [72]. Activation of Akt is hindered until it binds to either PI(3,4,5)P3 and PI(3,4)P2

through its PH-domain which is required for full enzymatic activity [59]. It was also suggested through affinity binding analysis that Akt has a higher binding affinity to PI(3,4)P2 and full activation of Akt was achieved after PI(3,4)P2 peaked at approximately 60sec [59]. Deactivation of Akt and its upstream kinases is dependent on the turnover of PI(3,4,5)P3 and PI(3,4)P2 from the negative regulating roles of the PTEN and INPP4 phosphatases [71].

The Akt branch of the PI3K pathway plays a pivotal role in B cell activation, differentiation, growth, cell cycle, cytokine production, survival, and metabolic regulation including glucose metabolism [26, 29]. The production of PI(3,4,5)P3 and PI(3,4)P2 during BCR stimulation is required for Akt to translocate to the PM and be fully phosphorylated by PDK1 and mTORC2 (**Fig. 5**). This binding and phosphorylation of Akt protects it from dephosphorylation which can only occur when Akt dissociates from the PIs [71]. In recent studies, it is demonstrated the phosphorylation at S473 has been correlated with PI(3,4)P2 levels and T308 phosphorylation with PI(3,4,5)P3 levels [58]. Two phosphatases have been implicated in the dephosphorylation and de-activation of Akt: protein phosphatase 2A (PP2A, dephosphorylates at T308) and PH-domain leucine-rich repeat protein phosphatases (PHLPP, dephosphorylates at S473). The mechanistic process on how these two phosphatases dephosphorylates Akt is still unclear [71].

Phosphoinositide-dependant kinase (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) are critical in the full activation of Akt and B cell activation in general. PDK1 is regarded as a master kinase as it not only phosphorylates Akt, but other downstream targets including p70-S6K, SGK, PCK, and RSK [73]. B cells that are deficient in PDK1 have been shown to have strong BCR signaling impairments [74]. PDK1 is a PI3K-dependent kinase as it needs the production of PI(3,4,5)P3 to bind its C-terminal PH-domain and become

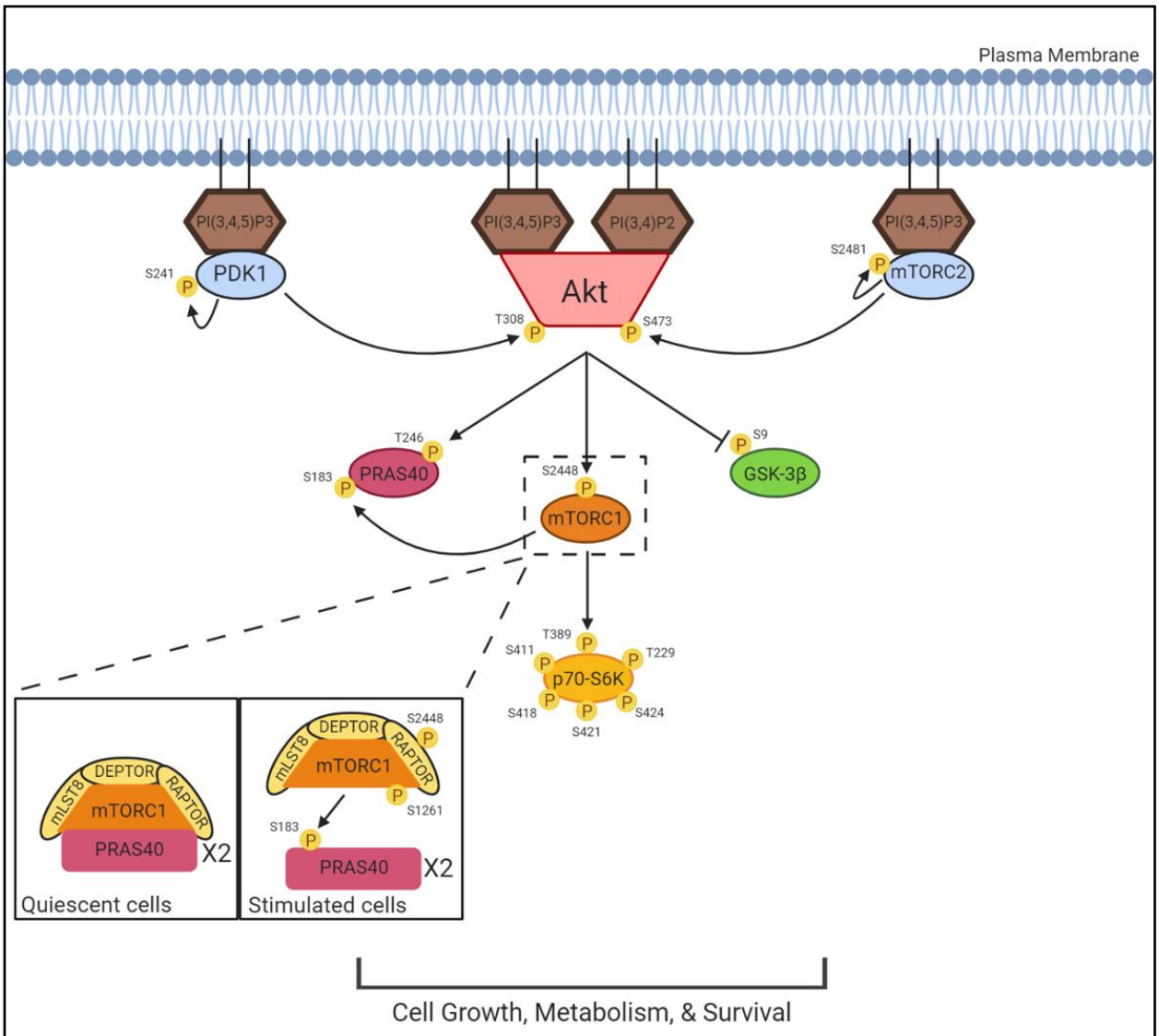


Figure 5. <u>PI(3,4)P2 and downstream targets – The Akt branch.</u>

Akt binds to both PI(3,4,5)P3 and PI(3,4)P2 at the PM produced from PI3K activation via its PH-domain, becoming fully activated. PDK1 initiates Akt phosphorylation in its active loop at T308. Akt becomes fully activated once mTORC2 phosphorylates Akt at S473 in its hydrophobic motif. Active Akt initiates phosphorylation of the downstream targets PRAS40 (T246), mTORC1 (S2448), GSK-3β (S9), and 70-S6K (T389). These downstream targets of interest are involved in various and distinct cellular functions for cell activation, primarily the control and maintenance of the actin cytoskeleton (mTORC1), induction of protein synthesis at the ribosome (p70-S6K), a regulator of mTOR and a negative regulator of autophagy (PRAS40), and energy metabolism (GSK-3β).

phosphorylated to function [75]. The phosphorylation of PDK1 at S241 in the active T loop is interesting as PDK1 auto-phosphorylates itself in trans for kinase activity with the PH-domain acting as an auto-inhibitor [73, 75]. This phosphorylated site is also difficult to dephosphorylate due to steric inaccessibility therefore the kinase is essentially always "active." Other factors such as homo- or heterodimerization fine-tunes PDK1 activation [76]. During the initial steps of cell stimulation, PDK1 and Akt translocate to the PM to the available PI's and form a complex in order to phosphorylate Akt [73]. This physical contact between the two kinases prompts a steric conformational change in Akt to allow PDK1 access to phosphorylation Akt's active loop at T308 (**Fig. 5**) [76]. This sets Akt up to become phosphorylated at its C-terminal hydrophobic motif on S473 by mTORC2 and therefore becoming fully activated [29, 74].

1.1.6.2 Bidirectional signaling between Akt and mTOR complexes

mTOR is a serine/threonine kinase that forms two distinct protein complexes that are distinct in function: mTORC1 and mTORC2 [77]. mTORC2 contains an mLST8 subunit and DEPTOR subunit much like mTORC1, however contains a Rictor (rapamycin insensitive companion of mTOR) and a Sin1-PH domain [77, 78]. Much like PDK1, mTORC2 controls cell proliferation and survival through the regulation of AGC family protein kinases, including Akt. It also regulates actin cytoskeleton formation and cell migration through the phosphorylation of PKC [77]. The Sin1-PH domain of mTORC2 inhibits its catalytic ability. This strict binding to PI(3,4,5)P3 releases the block on mTORC2 activity to phosphorylate Akt at S473 but only in conditions of extracellular stimuli [78]. This critical phosphorylation step permits Akt to phosphorylate pivotal downstream targets including mTORC1, p70-S6K (indirectly), PRAS40, and GSK-3β. mTORC2 itself is predominately phosphorylated at S2481 through

autophosphorylation in its Rictor domain acting as a positive feedback loop allowing Akt phosphorylation at S473 (**Fig. 5**) [77, 79].

mTORC1 is a distinct protein complex whose main function is to initiate downstream cell growth, macromolecule biosynthesis, translational processes, and cytoskeleton arrangement to facilitate mobility [26, 34]. mTORC1 is similar to mTORC2 as is contains the DEPTOR and mLST8 domains, but instead of a Rictor domain it has a Raptor (regulatory protein associated with mTOR) domain and two inhibitory PRAS40 subunits instead of a Sin1-PH domain, making it a cytoplasmic mTOR complex (**Fig. 5 close up**) [77]. Akt modulates the phosphorylation of mTORC1 by inhibiting TSC and permitting Rheb to activate mTORC1. This then allows mTORC1 to phosphorylate PRAS40 at S183 to be released in order for PRAS40 to be further phosphorylated by Akt at T246 for full activation (**Fig. 5**) [80, 81]. S2448 is the predominate site of phosphorylation by Akt and/or p70-S6K on mTORC1 at the Raptor domain [79]. Phosphorylated mTORC1 activates further downstream targets, more importantly eIF4E-binding protein 1 (4EBP1) and p70-S6K (**Fig. 5**). These two downstream signaling are pivotal for controlling various subunits of eukaryotic translation initiation factors 4 and the recruitment of the 40S ribosomal unit, respectively, for 5' cap-dependent translation of mRNA in cell proliferation, migration, and invasion [81, 82]. This makes mTORC1 a critical step in the activation of B cells during BCR-ligation. Its downstream targets are involved in lipid and nucleotide synthesis, glucose metabolism, inhibiting autophagy, regulating protein turnover, and protein synthesis (largely from p70-S6K and 4EBP1 phosphorylation) [77].

70kDa ribosomal protein S6 kinase (p70-S6K) is a dual pathway kinase directly downstream of mTORC1. It regulates cell growth through the ribosomal subunit S6 substrate and cell survival by inhibiting the mitochondrial pro-apoptotic molecule BAD [83]. Its role in regulating

the 40S ribosomal protein S6 is vital as mutations in any of the various phosphorylation sites severely compromises the ability of the cell to progress through the G1 cell cycle phase [84]. In cell quiescence, p70-S6K interacts with eukaryotic initiation factor 3 (eIF3) to stay inactivated. mTORC1 activation associates with the eIF3 complex allowing the dissociation of p70-S6K [85, 86]. Despite the fact that p70-S6K is not a direct target of Akt activity but rather mTORC1, studies have shown Akt still has an effect on p70-S6K activation and overall function as hyperactivation of Akt results in hyperactivation of p70-S6K [84].

The proline-rich Akt substrate of 40kDa (PRAS40) has a unique role in the PI3K cell activation pathway as it is a substrate of Akt, and a negative regulating component/substrate of mTORC1 (**Fig. 5**) [80, 87]. This places PRAS40 at the intersection between the Akt and the mTOR signaling pathways [88]. PRAS40 preferably binds to the mTORC1 complex as it contains a Raptor domain (**Fig. 5 close up**) [87]. It has also been observed that PRAS40 may have a surprising mTOR- independent pro-apoptotic role, or other roles in cell survivability including supressing p53-mediated cellular senescence, the induction of transformed cell metastasis, immunoregulation especially through mTOR in various immune cells, and protein degradation by initiating the assembly of β-subunits of immunoproteasomes [88]. When PRAS40 is phosphorylated by mTORC1, this causes the dissociation of PRAS40 from mTORC1 and frees docking sites for p70-S6K and 4EBP1 [80, 87]. This dissociation from mTORC1 frees PRAS40 to be phosphorylated by Akt at T246 and bind to the 14-3-3 chaperon proteins, which aid in the regulation of cell apoptosis, proliferation, differentiation, adhesion, and survival (**Fig. 5**) [80, 89].

1.1.6.3 Akt/mTOR signaling effects on cell metabolism

Glycolysis is a common indicator of elevated metabolic activity in cancerous cells and is known to be regulated by the PI3K pathway [90]. In various B cell-derived cancers, glycolysis is favoured, and it shown to be a result from increased glucose uptake, transcription of glucose transporters (ex. Glut1) and hexokinase (HK2) to increase glucose imports and flux, and glycolytic enzymes, even in conditions where other metabolic pathways are preferable. This is known as the Warburg effect [26, 91, 92]. Intermediates produced during glycolysis act as precursors and are utilized by other biosynthesis pathways needed for the enhanced rate of cell grow and maintenance [93]. A recent review indicated PI3K/Akt drive glycolysis and that Myc (a proto-oncogene) drives both mitochondrial function and glycolysis and found to have a collaborative effect in promoting tumorigenesis in Burkitt's lymphoma [92]. In adaptive immune cell impairments, is has been shown Akt/mTORC1 signaling can affect the expression of glucose transporters (Glut1) and HK2 and therefore the levels of glucose uptake in the cells, with an increase in Akt signaling typically associated with an increase in glycolytic metabolite levels [92, 94].

1.1.6.4 Akt and GSK3

Glycogen synthase kinase 3 (GSK-3) is a serine-threonine kinase comprised of two isoforms, GSK-3α and GSK-3β, with over 100 known substrates reported recently [95, 96]. GSK-3 is a fundamental kinase in metabolic pathways as it has an inhibitory affect on downstream substrates when active, helping maintain metabolic quiescence. Akt modulates metabolic activity by partially inhibiting GSK-3β via the phosphorylation at the S9 site **(Fig. 5)** [26]. GSK-3β is involved in energy metabolism control through glycogen metabolism as its name suggests [97]. It has also been described to have a wide spectrum of roles like

inflammation, ER-stress, cell division, stem cell renewal, mitochondrial dysfunction, apoptotic pathways, cell differentiation, transcription, and more [96, 98].

1.1.6.5 Disease associated with Akt

Since Akt is a pivotal checkpoint for various downstream processes during cellular activation, dysregulation of Akt can result in cellular transformations and disease. Akt hyperactivation is a common event in cancers [71]. Typically, Akt is hyperactivated by no fault of its own, but rather through deficiencies that negatively regulate PI3K activation, such as PTEN mutation, causing an increase in PI(3,4,5)P3 and PI(3,4)P2 levels for Akt to bind [67]. Over-activation of PI3K resulting from hyperactivation of tyrosine kinase receptors or mutations in the p110α catalytic subunit can also lead to an increase of Akt phosphorylation [34, 99]. Moreover, hyperactivation of Akt could be caused by a mutation within its PH-domain to allow Akt recruitment and binding to PI(4,5)P2, causing cancers [71, 99]. In many cancers, glycolysis is increased due to the rapid proliferation of cells [90]. Akt regulates various processes in glucose metabolism therefore hyperactivation of Akt enhances glucose metabolism via increased induction of HK2 and oxidative phosphorylation through the regulation of mTORC1 and GSK-3 [26, 72].

Deficiency in Akt also has devastating cellular consequences. In B cells, a decrease in Akt activation leads to a decrease in B cell subsets including MZ-like cells, memory B cells, and plasmablasts in common variable immunodeficiency patients. These patients have a lower numbers of membrane Ig-bearing B cells and have worse clinical outcomes overall [29, 100]. Another fact to note is Akt1, Akt2, and Akt3 react differently when deficient. For instance, Akt1 deficiency in $PTEN^{+/-}$ cells are able to inhibit cancer cell proliferation in mice, whereas Akt2 and Akt3 deficiency in the same $PTEN^{+/-}$ cells will show decreased or increase tumorigenesis,

respectively, in mice [99]. Therefore, targeting cancerous cells with Akt dysregulation is difficult.

In recent years, treatments options have been developed to eradicate cancerous cells by specifically targeting Akt or upstream/downstream proteins of Akt. For example, in follicular lymphoma cases with an increase in Akt S473 phosphorylation, treatment with a dual upstream PDK1/mTORC2 kinase inhibitor (NVP-BEZ235) showed anti-tumor activity and inhibited cell proliferation in xenograft mice models [26]. Rapamycin has been used to inhibit the excessive mTOR activation resulting from Akt hyperactivation and inhibits tumor development in xenograft prostate cancer mouse models when combined with other therapeutic treatments [72]. In B cell cancers, treatment with PI3Kδ inhibitor Idelalisib (Cal101) promotes cell death as Akt activation is decreased and pro-apoptotic factors are no longer inhibited [26, 101]. Currently, there are pan-Akt inhibitors being tested in clinical trails for cancer therapy including GSK690693, GDC0068, AZD5363 and the allosteric inhibitor MK-2206, which is beneficial considering the different functions of the Akt isoforms mentioned above [72, 99]. The main issue surrounding utilizing pan-Akt inhibitors (and pan-PI3K inhibitors in the same regard) are unwanted side effects – the most common being hyperglycaemia and hyperinsulinemia [72, 99]. Moreover, it has been unfortunately discovered that the use of pan-Akt inhibitor makes patients more susceptible to liver damage, cancer, and inflammation during treatment of their current cancer [99]. More research is needed on the negative effects of cancer treatments using pan-Akt inhibitors or other indirect treatments in order to control Akt activity.

1.2 Structure and regulation of INPP4

1.2.1 Molecular characteristics

INPP4 was first discovered in 1987 by the group Bansal *et al.* (1987) when they realized a novel 4-phosphatase was able to hydrolyse Ins(3,4)P2 to Ins(3)P in calf brain supernatant [102]. INPP4 was characterized biochemically to be approximately 105-110kDa, monomeric, have the ability to hydrolysis the D-4 phosphate from Ins(1,3,4)P3 and Ins(3,4)P2 in an Mg^{+}-independent manner, and is not inhibited by Li^{+}. These characteristics differ to previously described phosphatases such as the Mg^{+}-dependent inositol polyphosphate 1-phosphatase and SHIP1 [40, 103]. This 4-phosphatase is highly conserved as cDNA and amino acid sequencing were showed to be near identical (90% coding nucleotide and 97% amino acid similarity) between purified INPP4 samples gathered from human and rat brains. This conservation indicated essential functions within cells [104]. INPP4 is substrate specific as it binds and hydrolyses Ins(1,3,4)P3, Ins(3,4)P2, and PI(3,4)P2 but not other inositol phospholipids with a phosphate at the D-4 position including Ins(4)P, Ins(1,4)P2, Ins(1,4,5)P3, Ins(1,3,4,5)P4, or PI(3,4,5)P3 [48, 103]. It has also been suggested that INPP4 has phospho-tyrosine, phospho-serine, and phospho-threonine phosphatase activity [105]. However, it was later discovered by kinetic assays that INPP4 has a substrate affinity to PI(3,4)P2 120-fold and 900-fold greater than Ins(3,4)P2 and Ins(1,3,4)P3, respectively [48].

As more genetic research was conducted on this 4-phosphatase, the group Norris *et al.* (1997) discovered a distinct cDNA encoded for a 4-phosphatase which was also approximately 105kDa in size though it was only 37% identical at the amino acid level to INPP4; this was later denoted as INPP4B with the original 4-phosphatase labelled INPP4A [51]. INPP4B is also conserved between species as it has a 90% identical amino acid sequence between rat and humans [51].

These two isoforms have similar phosphatase activity as they both contained the conserved phosphatase activity sequence CKSAKDR (i.e. CX_5R) to hydrolyze PI(3,4)P2 in a time- and concentration-dependent manner [48, 51, 106]. This catalytic site is not only conserved between other 4-phosphatases that preferably hydrolyses the D-4 phosphate of PI(4,5)P2, but also between species such as nematodes and drosophila [107, 108]. Even between other PI phosphatases there is a degree of analogy in the conserved catalytic site. For example, the 3-phsophatase PTEN catalytic site (CKAGKR) is 25% similar to INPP4 (CKSAKDR), with the cysteine and the lysine's unchanged [108].

The basic structure of INPP4 is comprised of N-terminal tandem C2 domains, a PEST sequence, and a C-terminal catalytic domain (**Fig. 6A**) [49, 50, 109]. The C-terminal domain is where the phosphatase activity takes place in INPP4A/B within the CX_5R site. The D-4 phosphate of PI(3,4)P2 is hydrolysed by the nucleophilic catalytic cystine within this conserved site to PI(3)P (**Fig. 2 and 6A**) [49]. INPP4A and INPP4B can undergo various pre-mRNA and mRNA splicing resulting in differing C-terminal ends (Types Iα, Iβ, IIα, and IIβ) after being transcribed from the INPP4 type I (INPP4A) and INPP4 type II (INPP4B) genes located on chromosomes 2q11.2 and 4q28.1-q31.1, respectively [38]. These variations will result in the C-terminal end being either hydrophilic (Types Iα and IIα) or hydrophobic (Types Iβ and IIβ) [38]. The additional hydrophobic region at the C-terminus of the beta-forms of INPP4 results in its lack of enzymatic activity [41].

PEST sequences are found in several important proteins in various signal transduction pathways and marks these proteins as substrates for the calcium dependent thiol protease calpain [49]. Since INPP4A and INPP4B only have a 37% homology, one of these differences is INPP4B does not contain the PEST sequence (**Fig. 6A**) [51]. Therefore, calpain is seen as a

regulator of INPP4A as it decreases INPP4A activity through cleavage thus increasing overall PI(3,4)P2 levels in cells [49]. However, there may be a feedback loop to regulate calpain protease activity as the increase in PI(3,4)P2 levels inhibit calpain [49].

The N-terminal tandem C2-domains in the INPP4 structure appear to have dual functions in lipid binding and phosphatase activity based on consequences arising from mutations to specific amino acids (**Fig. 6A**) [50, 109]. C2-domains are involved in lipid interactions by basic residues and electrostatic interactions. Mutations to the negatively charged amino acids in the binding loops of the INPP4 type Iα C2-domains caused an enhancement of binding to the negatively charged lipids. Mutation analysis also observed the positively charged K122 and R124 amino acids in the lipid binding loops of C2-domain are involved lipid binding [50]. In the second and third binding regions (CBR2 and CBR3), the negatively charged amino acids D61, D120, and D123 coordinate calcium in other C2 domains by electrostatic binding. However, in lipid pull down assays it was shown that this calcium is not involved in lipid binding, it is instead shown to regulate the stability and phosphatase activity of INPP4 in human platelets [50]. C2-domains C2A and C2B bind to PI(3,4)P2 as simultaneous mutations occurring in both these domains displayed a loss of phosphatase activity of INPP4 Type Iα and loss in membrane recruitment. If one of these domains are still intact then binding is not affected [109]. On the N-terminus there is also an acidic arginine that is required for phosphatase activity and when mutated incited a complete loss of phosphatase activity and increased PI(3,4)P2 levels recorded [109].

Though the two isoforms of INPP4 function similarly and hydrolyse the same inositol lipid substrates, their respective expression levels in specific tissues may vary. For example, INPP4A was observed to have higher expression levels in the brain compared to the heart, skeletal muscle, spleen, kidneys, lungs, and testis while INPP4B expression are highest in the skeletal

muscles and heart relative to brain, placenta, pancreas, liver, kidneys, and lungs [49, 104]. The varying INPP4 isoforms subsets also has tissue-specific expression. For example, INPP4 Type Iα is a major form in platelets regulating the PI3K activity as well oxidative bursts in human neutrophils; INPP4 Type $I\alpha_3$ (a splicing variant of Type Iα) was found in mouse spleen, heart, lung, and uterus [38, 50]. INPP4 Type IIα was determined to be expressed in osteoclast (OC) and bone forming osteoblast (OB) cells [57]. These tissue-dependent expressions of specific INPP4 isoforms indicate that INPP4 could have tissue specific functions even though INPP4 is ubiquitously expressed in the body.

While interactions between INPP4 and other proteins have not been extensively examined, one study in human platelet cytosol suggested that a complex formed with the p85 regulatory subunit of Class I PI3K that helps INPP4 to localize to site of PI(3,4)P2 production during BCR-stimulation [106].

1.2.2 Functions of INPP4

Later research began describing alternative functions of INPP4A and INPP4B within certain tissues and their consequences when dysregulated. INPP4A was described to have a vital role during fetal brain development. In homozygous embryonic Weeble mice, which carry a spontaneous mutation within the INPP4A gene, neural defects were observed including cerebellum and hippocampus loss and severe ataxia. Neonatal death occurs in these mice due to neurons being sensitized to glutamate-mediated cell death [109, 110]. Neurological disorders such as temporal lobe epilepsy and Schizophrenia were linked to reduced INPP4A levels or chromosomal gene region deletion, respectively [41, 110].

INPP4B on the other hand was described as a tumor suppressor in breast cancer cells by regulating the duration of Akt activation and subsequently cell growth, metabolism and survival

[52, 53]. Normal Akt activation was obtained with the restoration of INPP4B [52, 53]. Mice models overexpressing INPP4B with xenografts of tumors showed a decrease in tumor size because Akt was not hyperactivated [52]. In breast cancer and some ovarian cancers, the INPP4B gene is commonly deleted (84%) together with other genes like breast cancer 1 (BRCA1) gene giving rise to overall poor patient survival [38, 52, 53]. Loss of INPP4B activity individually is usually not efficient in spontaneous tumorigenesis in some cell contexts, as seen in INPP4B$^{-/-}$ mice models [105]. Aggressive tumorigenesis with loss of INPP4B is commonly associated with loss of PTEN [41, 53, 110].

Recent studies have now indicated that INPP4B function might be a double-edge sword as there are cases in different cancer types that show that INPP4B can actually serve as an oncogenic driver, rather than a suppressor [55, 111]. Mass spectrometry profiling of acute myeloid leukemia (AML) showed significant increase in INPP4B expression and was associated with reduced sensitivity to DNA-damaging agents, early relapse, and poor survival [55, 111]. Interestingly, this oncogenic driving and chemoresistance function of INPP4B in AML seems to be PI phosphatase independent, as there was no correlation with Akt phosphorylation which the opposite was seen in other cancers [55]. Studies looking at a subset of melanomas have shown evidence that INPP4B overexpression can cause oncogenic signals and cell proliferation in a PI3K independent manner through serum- and glucocorticoid-regulated kinase 3 (SGK3) [54].

Within the last few years, novel research began arising on INPP4 having functions related to inflammation and immune cells. INPP4A acts as an asthma suppressor due to its PEST sequence and its expression in the lungs [46, 58]. Decreased INPP4A expression was found to cause spontaneous airway hyperresponsiveness and asthma-like conditions. Increasing INPP4A expression in bronchial epithelial cells alleviated this phenotype [46]. The increase of reactive

oxidative species during asthma stimulation induced the accumulation of PI(3,4)P2 in platelets, fibroblasts, and B cells which may be what is attenuated by INPP4A to reduce airway inflammation [58].

INPP4A colocalizes to endosomes and phagosomes and multiple studies determined that INPP4A acts as a positive regulator of clathrin-mediated endocytosis and a negative regulator of phagocytosis [50, 109, 112]. INPP4A colocalizes to early/recycled endosomes with the accumulation of PI(3,4)P2 and PI(3)P during development shown using fluorescent antibodies and localization microscopy methods. INPP4A has been demonstrated to be a negative regulator of phagocytosis as macrophages with INPP4A-specific knockouts having an increase in phagocytic activity and an increase in IgG-opsonized target uptake [112]. In INPP4A-specific knockout macrophages mice models, Akt hyperactivation was observed, as well as a decrease in inflammatory cytokines IL-12, IL-23, p40, and TNFα, and impaired bacterial clearance leading to endotoxemia/sepsis and a decrease in survival [56]. The PI3K/Akt pathway regulates the inflammatory response during infection by negatively regulating the production of pro-inflammatory cytokines while simultaneously positively regulating the production of anti-inflammatory cytokines to prevent accidental self-damage, with INPP4A negatively regulating this pathway [56].

Based on information gathered on INPP4 function in various cell types and the consequence when dysregulated, it appears likely that INPP4 may also be a critical regulator in the adaptive immune system through the PI3K pathway [20]. In 2013, a review summarized the roles of the inositol polyphosphatases within adaptive immune T cell differentiation, development, and functionality, with the roles of SHIP and PTEN in the context of PI(3,4,5)P3 explained in great detail. However, the authors acknowledged there is a knowledge gap of the function of INPP4 in

adaptive immune cells such as T and B cells [62]. Prior to that, in 2009 a review noted the inhibitory function of INPP4A in preventing the PI(3,4)P2-specific binding of TAPP2 during BCR-ligation when INPP4A was expressed at higher levels [20].

Previous work from our lab demonstrated the function of INPP4 in restricting the migratory capacity of cancer B cells [69]. It was observed in both B cell lines and primary CLL cell lines an increase in INPP4A activity depleted PI(3,4)P2 levels and attenuated chemokine-induced migration compared to the same cells overexpressing an inactivated version of the 4-phosphatase. They further showed that overexpression of INPP4A and the depletion of PI(3,4)P2 impaired the recruitment of cytoskeletal regulator Lpd to the migrating front of the B cells during cell stimulation with chemokines and therefore affected the motility and directionality of migration [69]. The opposite effect was reported when INPP4B expression levels were decreased, leading to increased migration in breast and melanoma cancer cells [69]. However, there has been no studies of the role of INPP4 in B cells activated via the BCR.

1.3 Summary of hypothesis, experimental approach, and results

1.3.1 Hypothesis, purpose, and questions

There has been a considerable amount of research on INPP4 in the last 30 years and more research is still being conducted on the affects INPP4 has on diseases that are devastating the everyday lives of individuals [82, 113-116]. However, as alluded to above there is limited understanding of how the affects of INPP4 on PI(3,4)P2 hydrolysis affects these specific downstream signaling pathways detailed. As well, no studies have examined the roles of INPP4 aids in lymphocyte activation, more specifically BCR activity in B cells. The purpose of my Thesis project is to fill the knowledge gap of how PI(3,4)P2 hydrolysis affects specific downstream signaling pathways and how INPP4 aids in B lymphocyte activation and regulation.

It is hypothesized that INPP4 regulates BCR signaling pathways and B cell metabolic activity through the control of PI(3,4)P2 levels via D-4 phosphatase activity. The specific questions related to this hypothesis are 1) how does INPP4 aid in the regulation of Akt phosphorylation, PM recruitment and phosphorylation of downstream targets, 2) does INPP4 regulate the Btk/PLCγ2/calcium axis, and 3) how does PI(3,4)P2 depletion by INPP4 affect B cell function, specifically metabolism?

1.3.2 Experimental approach

The interest in this Thesis is in the catalytic activity of INPP4 and the overall affects on PI(3,4)P2 levels on B cells during BCR signal transduction. In order to observe these effects, a controlled system was created to deplete PI(3,4)P2 levels. This was done by transducing a Burkitt's B lymphoma cell line (Bjab) with a 4v5-lentivirus containing vectors that overexpress either a functional (4v5-INPP4A WT) or a catalytically dead INPP4A gene (4v5-INPP4A mut). Various biochemical and analytical techniques were utilized to observe the effects that overexpressing active INPP4A would have on the levels of PI(3,4,5)P3 and PI(3,4)P2, activation/phosphorylation of Btk/PLCγ, Akt, and their downstream signaling targets, and the general effect on cellular metabolism.

1.3.3 Summary of results

Time-point stimulation assays revealed that INPP4A WT cells showed significant depletion of PI(3,4)P2 but no reproducible change in PI(3,4,5)P3 after BCR stimulation compared to INPP4A mut cells. Phosphorylation of Akt as well as some downstream targets such as GSK-3β and PRAS40 were also significantly impaired. Confocal microscopy examination of Akt-EGFP transfected INPP4A WT cells showed decreased PM recruitment compared to INPP4A mut cells. In contrast, active INPP4A had no effect on Btk or PLCγ phosphorylation, and calcium flux was

also not reduced. Metabolic glycolysis assays indicated that INPP4A WT cells have a significantly impaired ability to increase extracellular acidification rate after BCR stimulation compared to their mutant counterpart. These results show that depletion of one of the specific products of PI3K, PI(3,4)P2, can selectively affect the activation of some downstream phosphoinositide-binding proteins including Akt. Future experiments will further define the mechanisms by which PI(3,4)P2 depletion selectively affects these downstream signaling events and determine the impact on metabolic programming and B cells function *in vivo*.

Chapter 2: MATERIALS & METHODS

2.1 Materials

Human Bjab cells lines were originally obtained from the University of British Columbia (Vancouver, BC, Canada). pLenti4/V5-DEST INPP4A WT and pLenti4/V5-DEST INPP4A mut lentiviral vectors, and the plasmid Akt WT pEGFP-N were graciously gifted by Dr. Hongzhao Li (National Microbiology Laboratory, Winnipeg, MB, Canada). The pLenti-empty lentiviral vector was obtained from Addgene (Watertown, MA, USA) and the Akt WT plasmid was obtained by Clartech (Mountain View, CA, USA) [117]. Polybrene transfection reagent (cat. TR-1003-G) was obtained from EMD Millipore Corp. (Burlington, MA, USA), Zeocin (cat. R25001) from Invitrogen DNA (Carlsbad, CA, USA), and Puromycin (cat. A11138-03) from Gibco Cell Culture (Waltham, MA, USA). Neon transfection 10µL kit (cat. MPK1096) was purchased from Invitrogen (Carlsbad, CA, USA). HyClone^{TM} RPMI-1640 supplemented with 2.05mM L-Glutamine cell culture media (cat. SH30027.01) were ordered from GE Healthcare Life Sciences (Chicago, IL, USA). RPMI-1640 + GlutaMAX cell culture media (cat. 61870-

036), HBSS cell culture media (containing Ca^{2+} and Mg^{2+}, cat. 24020-117), and other cell culture components were ordered from Gibco Life Technologies (Waltham, MA, USA). The PIP3 Mass ELISA (cat. K-2500s) and PI(3,4)P_2 Mass ELISA (Cat. K-3800) kits were acquired from Echelon Biosciences (Salt Lake City, UT, USA). BioRad supplied the Quick Start™ Bovine Serum Albumin (BSA) standard set (cat. 500-0207) and the Protein Assay Dye Reagent Concentrate (5X) (cat. 500-0006) for protein quantification (Hercules, CA, USA). NP40 cell lysis buffer (cat. FNN0021) was ordered from Invitrogen (Carlsbad, CA, USA) along with the c0mplete Tablets, Mini EDTA-free, EASYpack Protease Inhibitor Cocktail Tablets (cat. 04-693-159-001) and PhosSTOP *EASYpack* Phosphatase Inhibitor Cocktail Tablets (cat. 04-906-837-001) from Roche (Basel, Switzerland). The Phospho(Ser473)/Total Akt Whole Cell Lysate kit (cat. K15100D-1) and the Akt Signaling (Total Protein) Panel Whole Cell Lysate Kit (cat. K15133D-1) multiplex panels, along with the mesoscale lysis buffer (phosphatase inhibitor I, Protease inhibitor solution, and phosphatase inhibitor II) and MSD Blocker A (cat. R93BA-4) was provided by Mesoscale Discovery (Rockville, MD, USA). Western blot supplies including the Mini-PROTEAN® TGX Stain-Free Gels 4-15% 10-well (cat. 456-8084) and 12-well (cat. 456-8085) gels, Precision Plus Protein™ Dual Color Standards (cat. 161-0374), and Clarity™ Western ECL Substrate (cat. 170-5061) for membrane development were all provided by BioRad (Hercules, CA, USA). Antibodies used for stimulation, western blots, mesoscale, and flow cytometry analysis were ordered from various companies listed in **Table 1**. Dapi, Dihydrochloride (cat. 28728-90-3) was ordered from Sigma Aldrich (St. Louis, MI, USA), Human BD Fc Block (cat. 564220) from BD Biosciences (San Jose, CA, USA), and Fluo-4 AM (cat. F23917) and Fura Red AM (cat. F3021) calcium probes from Life Technologies (Carlsbad, CA, USA). PI3K inhibitory drugs GDC-0980 (clinical name Apitolisib, cat. S2696) and Cal101

(clinical name Idelalisib, cat. S2226) were purchased from Selleck Chemicals (Houston, TX, USA), while the Syk inhibitor drug R406 (cat. S2194-5MG) was obtained from Cedarlane (Burlington, ON, Canada). The antibiotics kanamycin (cat. 11815024) was purchased from ThermoFisher Scientific (Waltham, MA, USA). QIAGEN Plasmid Maxi Kit (cat. 12163) and the Plasmid Buffer Set (cat. 19046, Hilden, Germany) were utilized to collect plasmids transformed in Subcloning Efficiency DH5α competent *Escherichia coli* (*E.coli*) cells (cat. 18265017) purchased from ThermoFisher Scientific (Waltham, MA, USA). The Neon Transfection system and reagents from the 10µL Neon Transfection kit (cat. MPK1096) were purchased from Life Technologies (Carlsbad, CA, USA). Corning® Cell-Tak™ and Tissue Adhesive (cat. 354240) was purchased from VWR Avantor (Radnor, PA, USA). The Agilent Seahorse XF Glycolytic Rate Assay Kit and the Seahorse XF 24 Analyzer were manufactured by Agilent (Santa Clara, CA, USA).

2.2 Cell culture

The Bjab human B lymphocyte are a human Burkitt lymphoma B cell line and are PTEN deficient [118]. Bjab cells were grown in RPMI-1640, 5-10% Fetal Bovine Serum (FBS), and 1% Penicillin/Streptomycin (PenStrep) complete media (HyClone GE Healthcare Life Sciences; Gibco). Cells were incubated at 5% CO_2 at 37°C in filter-capped flasks. The cells were seeded approximately 2.0-2.5x10^5 cells/mL per feeding for optimal growth.

Table 1. List of Antibodies

Name	Purpose	Catalog No.	Company	Address
Type I 4-phosphatase (A-19), goat polyclonal IgG	Western Blots	SC-12315	Santa Cruz BioTechnology	Dallas, TX, USA
P-PDK1 (S241) Rabbit mAb	Western Blots	3061S	Cell Signaling Technology	Danvers, MA, USA
PDK1 Rabbit Ab	Western Blots	3062S	Cell Signaling Technology	Danvers, MA, USA
Phospho-mTOR (Ser2448) Antibody	Western Blots	2971L	Cell Signaling Technology	Danvers, MA, USA
mTOR (7C10) rabbit mAB	Western Blots	2983S	Cell Signaling Technology	Danvers, MA, USA
P-Btk (Y223) Rabbit Ab	Western Blots	5082S	Cell Signaling Technology	Danvers, MA, USA
Btk (C82B8) Rabbit mAb	Western Blots	3533S	Cell Signaling Technology	Danvers, MA, USA
Phospho-PRAS40 (T246) Rabbit Ab	Western Blots	2640S	Cell Signaling Technology	Danvers, MA, USA
PRAS40 Rabbit Ab	Western Blots	2610S	Cell Signaling Technology	Danvers, MA, USA
beta-actin (13E5) rabbit mAb	Western Blots	4970S	Cell Signaling Technology	Danvers, MA, USA
Anti-Cyclophilin B antibody [EPR12703(B)] – Loading Control	Western Blots	ab178397	Abcam	Cambridge, United Kingdom
Anti-goat IgG, HRP-linked secondary Ab	Western Blots	705-035-003	Jackson ImmunoResearch Laboratories, Inc.	West Grove, PA, USA
Anti-rabbit IgG, HRP-linked secondary Ab	Western Blots	7074S	Cell Signaling Technology	Danvers, MA, USA
Goat F(ab')$_2$ anti-human IgM (μ) Alexa Fluor 647	BCR Overexpression	109-606-129	Jackson ImmunoResearch Laboratories, Inc.	West Grove, PA, USA
Goat F(ab')$_2$ Anti-human IgM-UNLB (0.5mg/mL)	Stimulation	2022-01	Southern Biotech	Birmingham, AL, USA
Goat Anti-Human IgM-UNLB (1mg/mL)	Stimulation	2020-01	Southern Biotech	Birmingham, AL, USA
Alexa Fluor® 647 Mouse anti-PLC-γ2 (pY759)	PLCγ phospho-flow cytometry	558498	BD Biosciences	San Jose, CA, USA
p-Akt (Ser473)	Mesoscale	K15100D-1	Mesoscale Discovery	Rockville, MD, USA
Akt	Mesoscale	K15133D-1	Mesoscale Discovery	Rockville, MD, USA
p-p70S6K (Thr421/Ser424)	Mesoscale	K15100D-1	Mesoscale Discovery	Rockville, MD, USA
p70S6K	Mesoscale	K15133D-1	Mesoscale Discovery	Rockville, MD, USA
p-GSK-3β (Ser9)	Mesoscale	K15100D-1	Mesoscale Discovery	Rockville, MD, USA
GSK-3β	Mesoscale	K15133D-1	Mesoscale Discovery	Rockville, MD, USA

2.3 Constructs

Wildtype (WT) or mutant (mut) version of the INPP4A vector sequence were inserted into the pLenti4/V5-DEST vector (Life Technologies) as described [69]. INPP4A mut was catalytically inactivated by point mutating the catalytic nucleophilic cysteine within the conserved phosphatase motif CKSAKDR to a serine (i.e. SKSAKDR) using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). This mutation nullified the phosphatase activity of INPP4A. Akt WT pEGFP-N plasmids were generated as described [117]. Phosphatase motif modifications and all plasmid constructs were verified by DNA sequencing (Robarts Research Institute, DNA Sequencing Facility, London, ON, Canada).

2.4 Lentiviral transduction

Bjab cells were transduced using lentiviruses containing the 4v5-INPP4A constructs gifted by Dr. Hongzhao Li. $5.0x10^4$ Bjab cells were collected, centrifuged, and resuspended in 250µL of supernatants containing pLenti-4v5-INPP4A or pLenti-4v5-INPP4A mut lentiviral constructs ($Zeocin^r$). Polybrene (EMD Millipore) was added (final concentration 8µg/mL) to the cell mixture and then centrifuged for 1hr at 800g at room temperature (RT). After drug selection using 2mg/mL Zeocin for approximately 14days, frozen stocks were prepared of the newly transduced cell lines.

2.5 Stock preparations

1mL stocks were created containing $5.0\text{-}10x10^6$ Bjab cells. A freezing solution containing 50% complete cell culture media (10% FBS and 1%PenStrep), 30% FBS, and 20% DMSO (Sigma-Aldrich) was prepared on ice. Cells were collected, centrifuged, and resuspended in complete cell culture media on ice. An equal part of freeze media was slowly added to the resuspended cells (final concentration of DMSO = 10%) and placed back on ice. The cells were

aliquoted equally and placed in -80°C and then transferred into liquid nitrogen the next day or a few days later. When cells were needed, they were thawed, centrifuged, resuspended in pre-warmed complete media with 20% FBS, and incubated in 37°C, 5% CO_2. The next day, they were returned to complete media containing between 5-10% FBS.

2.6 Cell lysis

2.6.1 NP40 lysis

Cells lysis with NP40 lysis buffer proceeded with the provided NP40 Cell Lysis Buffer protocol (Invitrogen) with a few modifications. NP40 lysis buffer was freshly made with PMSF and protease inhibiter cocktail (Roche). $5.0x10^6$ cells were collected from each cell line and resuspended in cold PBS, and centrifuged. Cells were resuspended in 75µL lysis buffer for 30min on ice, vortexing at 10min intervals. Protein lysates were centrifuged at maximum speed (17,000 x g) at 4°C and supernatants collected and stored in -80°C.

2.6.2 Mesoscale lysis

After cell stimulation (see below), cells were lysed with mesoscale lysis buffer from the Phospho-Akt (Ser473)/Total Akt Whole Cell Lysate Kit (MSD Multi-Array Assay System) using a similar protocol used for NP40 lysis. Cells were lysed in 150µL of mesoscale lysis buffer as the lysates were used in both mesoscale assays and western blot analysis. After a 10min incubation in the lysis buffer, protein lysates were centrifuged at maximum speed (17,000 x g) at 4°C then supernatants collected and stored in -80°C.

2.7 Protein quantification

2.7.1 BCA protein assay

The Microplate Procedure was utilized from the Pierce BCA Protein Assay Kit (Thermo Scientific) with slight modifications. 1mg/mL, 0.5mg/mL, 0.25mg/mL, 0.125mg/mL, and 0.0625mg/mL standards, and a ddH_2O blank were pipetted in the 96-well plate in triplicates. Sample lysates were diluted 1:4 and 10µL of samples were pipetted in triplicates. 200µL of Working Buffer (50:1, Reagent A:B) was added to each well for a sample to working reagent ratio equaling 1:20. Plates were incubated in 37°C at 5% CO_2 for 30min, covered, and then cooled. Plates were read at 562nm with the SoftMax Pro Spectrometer and software version 5.4.1 (Molecular Devices, San Jose, CA, USA).

2.7.2 BioRad protein quantification procedure

2mg/mL, 1.50mg/mL, 1mg/mL, 0.75mg/mL, 0.50mg/mL, 0.25mg/mL, 0.125mg/mL, and 0.125mg/mL standards, along with a blank were pipetted in duplicate in a 96-well plate. 250µL prepared dye reagent from the BioRad Protein Quantification Assay was pipetted onto the standards and 2µL undiluted protein lysate samples. Plates were read on SoftMax Pro Spectrometer (Molecular Devices) at 595nm. Other settings included Dilution Factor = 2.5 and Other = unknown[Dilutions].

Protein concentrations were calculated from the absorbance values gathered from both methods of protein quantification assays.

2.8 INPP4A WT and INPP4A mut overexpression verification

20µg of protein from the parental/non-transduced and transduced Bjab cell lines were ran through electrophoresis on Mini-PROTEAN® TGX Stain-Free Gels 4-15% western blot gels (BioRad) and wet transferred onto membranes (transfer buffer: 25mM Tris, 190mM glycine, 20% methanol). Membranes were incubated overnight in 4°C with Type I 4-phosphatase (A-19) goat polyclonal IgG (i.e. INPP4A, 1:1000) (Santa Cruz BioTechnology) and β-actin rabbit primary antibodies (1:5000) (Cell Signaling Technology) . The next day, membranes were washed and incubated with anti-goat and anti-rabbit HRP-linked secondary antibodies (1:1000, for INPP4A and β-Actin, respectively) for 1hr at RT. The membranes were developed with Clarity™ Western ECL Substrate (BioRad) and pictures taken using BioRad ChemiDoc™ MP chemiluminescence imaging system.

2.9 BCR expression verification

$5.0x10^5$ Bjab cells (parental and transduced) were collected each to be either stained or left unstained (control). Cells to be left unstained were resuspended in 200µL cold FACS buffer (cold 1x PBS with 2% FBS). Cells to be stained were resuspended in 200µL cold FACS buffer with Goat F(ab')2 anti-human IgM (µ) Alexa Fluor 647 (1:400 final dilution, Jackson ImmunoResearch Laboratories, Inc.) along with 1µL Human Fc Blocker (BD Biosciences). After incubation, all cell samples were washed and resuspended in FACS buffer. DNA stain Dapi (Sigma Aldrich) was added and stained cells were analyzed using a FACSCanto II flow cytometer. Live cells were differentiated from dead cells using Pacific Blue channel fluorescence from the Dapi staining and BCR expression was gated from the APC channel.

2.10 INPP4A phosphatase activity verification (Competitive ELISA)

The PIP_3 Mass ELISA (TDS K-2500s) kit and $PI(3,4)P_2$ Mass ELISA kit (TDS K-3800) from Echelon Biosciences were used according the manufacturers protocols. Stimulation controls were pre-treated with 1µM GDC-0980 or 5µM Cal101 PI3K inhibitors for 30min prior to stimulation. Lipids were extracted after cells (Bjab parental, and 4v5-INPP4A WT and mut transductants) were stimulated for 0min, 0.5min, 1min, 1min+Cal101, and 1min+GDC-0980 (PIP3 assay), or 0min, 1min, 2min, 5min, 15min, 5min+GDC-0980, and 5min+Cal101 ($PI(3,4)P_2$ assay) with 2 or 10µg Goat Anti-Human IgM-UNLB (Southern Biotech).

Used 2-way ANOVA t-test for PI(3,4)P2 and PI(3,4,5)P3 for statistical analysis. The column factor is the Time (1min vs. 2min vs. 5min vs. 15min or 30sec vs. 60sec) and the row factor is the Genotype (4v5-INPP4A WT vs. 4v5-INPP4A mut). P-values ≤ 0.05 were considered statistically significant.

2.11 Cell stimulation and phosphorylation assays

2.11.1 Cell stimulation

$1.0x10^7$ Bjab transduced cells were collected per time-point condition. Cells were stimulated for 0min, 2min, 5min, 15min, 30min, and 15min+GDC-0980 PI3K inhibitor with 2µg/mL anti-human $F(ab')_2$ IgM antibody (Southern Biotech). Cells were serum starved in empty pre-warmed RPMI media ($1.0x10^7$ cells/500µL) for 3hrs in 37°C at 5% CO_2, gently shaking every hour to resuspend cells. In the last 30min, the pan-PI3K inhibitor GDC-0980 (Selleck Chemicals) was added (final concentration of 2µM) to the subset of cells to be stimulated for 15min+GDC-0980. Stimulation time assay was conducted for the pre-determined time points with respective amount of $F(ab')_2$ anti-human IgM in the 37°C water bath. Immediately completing the stimulation assay, cells were lysed using mesoscale lysis buffer.

2.11.2 Western blot

15-20µg of protein, determined via the BioRad protein quantification, were loaded into Mini-PROTEAN® TGX Stain-Free Gels 4-15% western blot gels (BioRad) and ran for 2hrs at 100V. The proteins were wet transferred onto the membrane and immediately blocked with BSA blocking buffer (5% MSD Blocker A (Mesoscale Discovery) and TBST). Membranes were washed and cut at the respective protein sizes to separate the proteins of interest. Membranes were incubated overnight in their separate primary antibodies for INPP4A (1:1000), β-Actin (1:5000), and Cyclophilin (1:3000), and the following phosphorylated versions of the proteins: 1:1000 dilution pPDK1, p-mTOR, pBtk, and pPRAS40 (see **Table 1**). The next day the membranes were washed, and then incubated for 1hr at RT in either anti-goat (INPP4A) or anti-rabbit secondary antibodies (1:5000).

After image development, the phosphorylated protein antibodies (pPDK1, p-mTOR, pBtk, and pPRAS40) were stripped twice for 10min in glycine stripping buffer (2.2M glycine, 0.5M NaCl, pH 4.4). The newly stripped membranes were re-incubated with their represented total protein primary antibodies (1:1000 PDK1, mTOR, Btk, and PRAS40, see **Table 1**) overnight in 4°C. The membranes were developed in anti-rabbit secondary antibody the same way as mentioned previously.

2.11.3 Mesoscale

The Phospho(Ser473)/Total Akt Whole Cell Lysate kit (cat. K15100D-1) and the Akt Signaling (Total Protein) Panel Whole Cell Lysate Kit (cat. K15133D-1) multiplex panels (MSD MULTI-ARRAY Assay System) were utilized. The plates were prepared and analyzed in accordance to the protocol outlined and provided by the kits. MSD 96-well plates are coated with antibodies against the phosphorylated and total forms of Akt, GSK-3β, and p70-S6K on a

specific spot in the well for which the proteins in the lysate, collected by the MSD lysis protocol, would attach to. These spots are meant to detect various proteins in a sample. Afterwards, a secondary antibody conjugated with a biochemical marker (SULFO-TAG™ labeled detection antibody), would attach to the proteins of interest that were captured by the fixed antibodies in the wells.

2.11.4 Analysis and statistics

Individual membranes were analysed using ImageJ 1.51j8 software for western blots. Band Intensity values of each time point for each protein type (i.e. phosphorylated vs total protein) was obtained from the area underneath the curve of the band intensity in ImageJ. Mesoscale protein concentrations were obtained as outlined by the protocol. The relative ratios of phosphorylated protein:total protein was calculated for each time point and were normalized to the respective unstimulated time point of the 4v5-INPP4A mut samples. Statistical significance was determined in Prism version 7 (excluding the unstimulated and the 15min+GDC-0980 inhibitor time point) with a Two-Way ANOVA t-test test where the column factor is the stimulation time and the row factor is the cell genotype. P-values ≤ 0.05 were considered statistically significant.

2.12 Akt PH-domain PM recruitment assay

2.12.1 Plasmid preparation

The Akt WT pEGFP-N plasmid was transformed into Subclone Efficiency DH5α competent *E. coli* cells (ThermoFisher Scientific) using a modified version of the transformation protocol as described [119]. 1µL of plasmids were added to 20µL newly thawed competent DH5α *E. coli* cells and incubated on ice. The bacteria were heat shocked for 30sec in a 42°C water bath and 500µL of pre-warmed LB broth was added to each sample. After an hour-long shaking

incubation in 37°C, 20µL of newly transformed bacteria were spread plated on antibiotic infused LB agar plates (kanamycin, 1:1000) and incubated upside-down overnight at 37°C. The next morning (18hrs), an isolated transformed *E. coli* colony was incubated in pre-warmed 5mL LB broth with diluted antibiotic (1:1000), shaking at 37°C at 2500rpm and then later transferred to 150mL LB broth with diluted kanamycin antibiotics (1:1000) for overnight incubation. The next day, the plasmids were extracted.

Plasmids were extracted using the QIAGEN Plasmid Maxi Kit using the manufacturer's protocol they provided with a few alterations, primarily to the centrifuging speeds in step 5 (10,000 x g), step 10 (12,000 x g), and step 11 (7,500 x g) of the provided protocol. For the final step (step 12) after the ethanol was decanted from the DNA plasmids, the remaining ethanol was evaporated in a fume hood for 10min. Lastly, the DNA plasmids were redissolved in 300µL EB buffer or ddH_2O (rather than TE buffer suggested by the protocol). The concentration and the purity of the DNA plasmids were quantified using the NanoDrop Lite Spectrophotometer (Thermo Scientific) at factor 50. The extracted plasmids were considered acceptable when the A260/A280 ratio was between 1.80-2.00, indicating a relatively pure sample. The plasmids were stored at -20°C.

2.12.2 Transfection

Transfections were performed using the Neon Transfection system and reagents provided from the 10µL Neon Transfection kit (Life Technologies) with a slight modified version of the manufacturer's protocol. Suspended in 10µL homemade R buffer (1mM $MgCl_2$ and 250 mM sucrose in DPBS [Dulbecco's phosphate-buffered saline, Gibco cat. 14190], [120]), $5.0x10^5$ cells were transfected with 1µg of Akt WT pEGFP-N plasmids in optimized electroporation conditions for Bjab cells: one pulse at 1350V for a duration of 20ms. Newly transfected cells

were plated in 750µL plating/growth media (20% FBS, 100X HEPES, and 1X RPMI-1640+Glutamax) in 24-well plates and incubated for 18hrs at 37°C and 5% CO_2. Positively transfected cells were observed using the EVOS FL microscope (Life Technologies) and efficiency was determined via flow cytometry.

2.12.3 Cell stimulation and microscopy analysis

18hrs post-transfection, cells were starved in empty RMPI-1640 media for 1hr in 37°C at 5% CO_2 prior to cell stimulation. 2µM GDC-0980 pan-PI3K inhibitor (Selleck Chemicals) was added to each transfected cell line as controls in the last 30min of the serum starving. Cells were stimulated with 10µg/mL anti-human $F(ab')_2$ IgM (Southern BioTech) for 5min. After stimulation, cells were fixed by adding an equal volume of 4% PFA (2% final concentration). After 5min, cells were washed, resuspended and transferred in PBS into sterile 8-well microscope µ-slides (Ibidi, cat. 80826, Martinsried, Germany) to analyze PM recruitment through confocal microscopy (Zeiss, Oberkochen, Germany).

For each transfected cell, five mean fluorescent intensity values were taken at the PM and adjacent spots in the cytoplasm via ImageJ 1.51j8 software. Individual PM intensity/cytoplasm intensity ratios were calculated for each cell based on the average of these five values. The overall intensity ratio for the cell line and condition was obtained by averaging the PM/cyto ratios from approximately 25-30 cells per cell line and condition. Statistical analysis was performed on Graft Pad Prism7 using the Mann-Whitney test. P-values ≤ 0.05 were considered statistically significant.

2.13 Calcium assay

$5.0x10^6$ Bjab cells/tube were resuspended in pre-warmed RMPI media. 3.5µL Fluo-4 AM and 6.5µL Fura Red AM calcium probes (Life Technologies) were added to each tube. These fluorescent dyes were used to increase the calcium quantification accuracy since Fluo-4 (488nm red channel) fluorescence increases in the presence of Ca^{2+} while the fluorescent intensity of Fura Red (532nm green channel) is quenched in the presence of Ca^{2+} within cells [121, 122]. Cells were incubated, covered, for 30min at 37°C, shaking half way to ensure proper incubation and uptake of dyes. The cells were spun down, washed, resuspended in room temperature HBSS (Gibco) and transferred to FACS tubes for flow cytometry analysis. The FACSCanto II flow cytometer and gating were set up to observe the Fluo-4 AM (Alexa488) the Fura Red AM (PerPC) dyes. Samples were placed in the machine and collected cells for 30sec to determine the basal internal calcium levels prior to stimulation. After the 30sec, the tubes were ejected, and 10µg/mL Goat Anti-Human IgM (Southern Biotech) was added. The tubes were immediately placed back into the flow cytometer and collected for 7min.

Analysis of the kinetic Calcium Assays were conducted using FlowJo version 8.8.7. Live singlet cells were gated using FSC-H, FSC-A and SSC-A parameters. The internal calcium levels during BCR stimulation were quantified and displayed as a Fluo-4:Fura Red ratio on a linear scale. The newly derived parameter was created by multiplying Fluo-4 by 10 and then dividing that by Fura Red which was then all converted to 1:5 scale. The kinetic graphs were displayed as Calcium levels Ratio vs. Time (sec) using the median values (to lessen the weight of outliers) with moving average smoothing.

2.14 PLCγ2 phosphorylation assays (Intracellular flow cytometry)

$5.0x10^5$ cells were first serum starved in pre-warmed RPMI-1640 media for 2hrs. Cells were stimulated with 10μg/mL Goat $F(ab')_2$ Anti-human IgM (Southern Biotech) for 0min, 1min, 2min, 5min, and 15min. Cells were also pre-treated during the serum starvation with 1μM pan-PI3K inhibitor GDC-0980, 5μM PI3K p110δ inhibitor Cal101, or 10μM Syk inhibitor R406 for 30min and then stimulated for 5min. Cell fixing, permeabilization, and intracellular immunostaining was performed in accordance to the protocol provided by Cell Signaling [123]. Cells were stained/incubated with Alexa Fluor® 647 Mouse anti-PLC-γ2 (pY759) primary antibody (BD Biosciences, see **Table 1**). Phospho-PLCγ quantities were determined as mean fluorescence intensity in the Alexa647 channel, normalized to unstimulated 4v5-INPP4A mut. Statistical significance at individual time points were calculated using 2way ANOVA t-tests. P-values ≤ 0.05 were considered statistically significant.

2.15 Metabolic assay

The rate of glycolysis within cells was measured using a Seahorse XF 24 Flux Analyser (Agilent). Glycolysis is detected via the changes in media pH denoted as the extracellular acidification rate (ECAR) after glucose is injected into each well (final contraction of 5mM). The following protocol was specifically designed to test the glycolytic rate of suspended B cells in various conditions. It was modified from the Agilent protocol [124].

A day prior to the experiment, Bjab cells were seeded in fresh medium at a concentration of $2.0\text{-}2.5x10^5$ cells/mL and incubated at 37ºC in 5% CO_2. As well, an XF sensor cartridge was calibrated overnight at 37°C in an CO_2-free incubator. The next day, $5.0x10^6$ cells were washed in 3mL pre-warmed glucose-free XF assay media (unbuffered DMEM, pH = 7.35, filter sterilized using 0.22μm filter) supplemented with L-glutamine (final concentration = 2mM) and

then resuspended in 2.5mL of the same media ($2.0x10^3$ cells/μL). 100μL of the resuspended cells were immobilized to the wells of a XF analyser 24-well microplate coated with 1.12μg Cell-Tak (0.1M solution of sodium bicarbonate to a concentration of 22.4μg/mL per well, Corning, cat. 354240). To ensure adherence, the plates were centrifuged at RT at 1100rpm and then incubated in a 37°C CO_2-free incubator for maximum 20min, topped-off with 460μL of the glucose-free XF assay media supplemented with L-glutamine, and incubated for an additional 30min in the CO_2-free incubator. During this incubation time, the XF sensor cartridge that was calibrated overnight was prepared by adding 80μL of 8μM pan-PI3K inhibitor GDC-0980 (diluted in the supplemented XF assay media, final concentration of 1μM once injected) or a media blank (Port A), 80μL of 90μg/μL anti-human $F(ab')_2$ IgM (final concentration = 10μg/mL) for stimulated conditions or another blank for unstimulated conditions (Port B), 80μL of 50mM D-glucose solution (Port C for a final concertation of 5mM when injected in each well), and finally 80μL of 220mM 2-Deoxy-D-glucose solution (2-DG) (Port D for a final concentration of 20mM when injected in each well). After the addition of all solution into their respective ports, the cartridge was placed in the Seahorse XF 24 Analyzer along with the immobilized monolayered cells and rate of glycolysis recorded [124]. This measurement was outputted as ECAR through changes in pH levels. Average basal (measurements obtained pre-injections) and active glycolysis (immediately after D-glucose injection) were calculated from the three or four measurement points within their respective time ranges. Statistical significance was tested using parametric paired t-tests. P-values ≤ 0.05 were considered significant.

Chapter 3: RESULTS

3.1 Overexpression of active or inactive INPP4A in B lymphoma cells

To observe how depletion of PI(3,4)P2 levels affects downstream binding targets and signaling activation during BCR ligation, human Bjab and Raji B cell lines were transduced with a pLenti4/V5-DEST vectors inserted with either a wildtype or mutant version of INPP4A (4v5-INPP4A WT and 4v5-INPP4A mut, respectively). INPP4A mut was catalytically inactivated by point mutating the catalytic nucleophilic cysteine within the conserved phosphatase motif (**Fig. 6A**). Western blot analysis of non-stimulated parental (i.e. non-transduced B cells), 4v5-INPP4A WT, and 4v5-INPP4A mut Bjab and Raji cell lines was used to verify the overexpression of WT and mut INPP4A (**Fig. 6B**). Based on the similar high expression levels between INPP4A WT and INPP4A mut in 4v5-transduced Bjab cells (20x and 21x higher than Bjab parental, respectively), Bjab cells were utilized in all subsequent experimental analysis.

Post transduction, surface BCR expression was evaluated. This was to ensure the transduction processes did not alter the BCR expression levels as all proceeding experiments required BCR stimulation and activation of the PI3K signaling pathway. Cells were stained with Alexa-647-labeled anti-human IgM and analysed by flow cytometry. The level of BCR expression was displayed as levels of APC-A expression. Compared to the unstained Bjab parental negative control and stained Bjab parental positive control, Bjab 4v5-INPP4A WT and

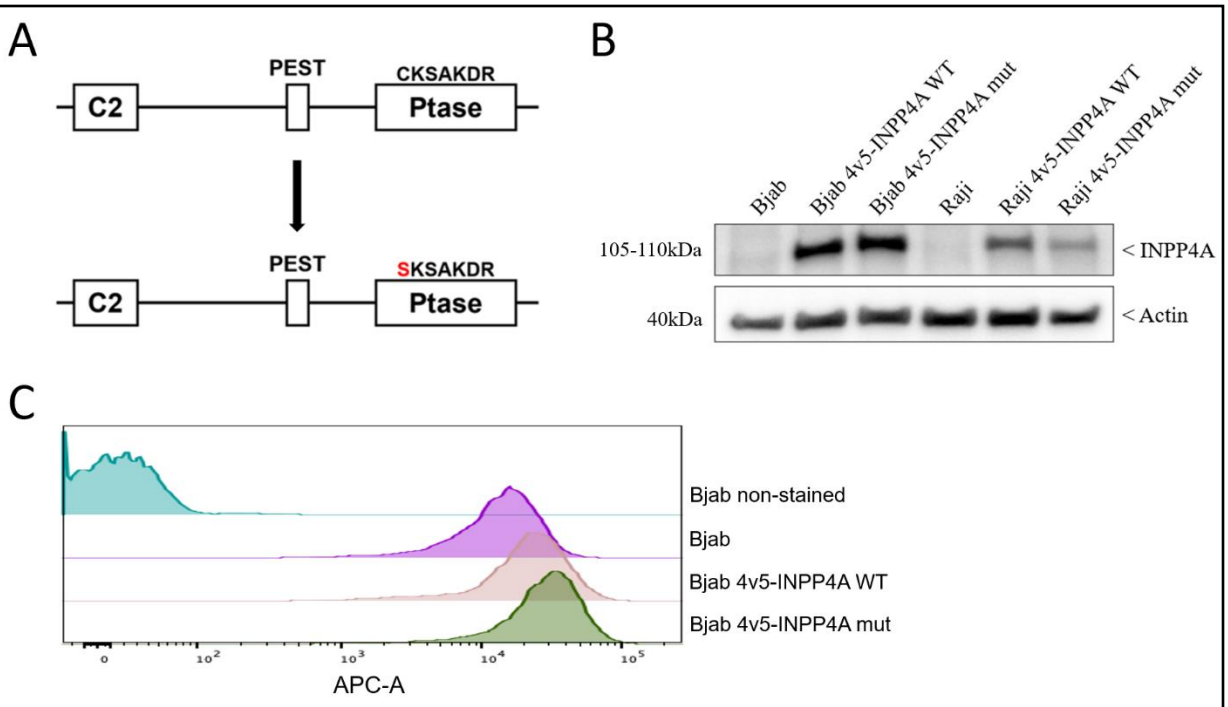


Figure 6. <u>WT and mut INPP4A successfully overexpressed with no negative effect on membrane BCR expression in transduced human B lymphoma cell lines.</u>

(A) Diagram illustrating INPP4A constructs with N-terminal tandem C2 domains, a PEST sequence, and the C-terminal phosphatase domain constructs inserted in pLenti4/V5-DEST lentiviral vectors. INPP4A mutant constructs were created by mutating the catalytic nucleophilic cysteine to a serine to nullify phosphatase activity. Modified from <u>Li & Marshall, 2015. *Phosphatidylinositol (3,4) bisphosphate-specific phosphatases and effector proteins: A distinct branch of PI3K signaling*. Cellular Signalling, 27 (1789-1798</u>). **(B)** Human B cell lines were transduced to overexpress either active INPP4A (4v5-INPP4A WT) or catalytically inactivated INPP4A (4v5-INPP4A mut). 4v5-INPP4A WT and mut overexpression in non-stimulated transduced Bjab and Raji B cell lines was confirmed by western blots, probed with Type I 4-phosphatase (A-19) primary anti-antibody against INPP4A (representative figure). **(C)** BCR expression verification of transduced Bjab cells by flow cytometry. Parental/untransduced and transduced cells were probed with Goat F(ab')2 anti-human IgM (μ) Alexa Flour 647. APC-A vs. SSC gated for BCR expression (representative figure).

Bjab 4v5-INPP4A mut transduced cells do not show any reduction of BCR levels post-transduction (**Fig. 6C**).

3.2 Impact of INPP4A overexpression on phosphoinositide levels

To verify that INPP4A WT overexpressed within Bjab cells is functioning to specifically hydrolyze PI(3,4)P2, competitive ELISA experiments were conducted to quantify the total amount of PI(3,4)P2 and PI(3,4,5)P3 lipids extracted from stimulated Bjab 4v5-INPP4A WT and mut cells. This was done by extracting the PI lipids from cells stimulated for the allotted time, incubating them with either a PI(3,4)P2- or PI(3,4,5)P3-binding detector protein and then adding the mix to the PI-coated plate. The signal obtained from the analysis is inversely proportional to the available lipid concentration competing for binding to the detector.

The lipids were extracted in accordance to the manufacturer's protocol after cells were stimulated for 0min, 0.5min, 1min, 1min+Cal101, and 1min+GDC-0980 for the PI(3,4,5)P3 analysis and 0min, 1min, 2min, 5min, and 15min, 5min+Cal101, and 5min+GDC-0980 for the PI(3,4)P2 analysis. The pan-PI3K inhibitor GDC-0980 or PI3Kδ inhibitor Cal101 were used as negative controls by shutting down PI3K activation fully or partially (**Fig. 7**). As expected, it was found that the levels of PI(3,4)P2 were reduced in cells overexpressing active INPP4A relative to cells expressing inactive INPP4A cells. This was the case for all time points, including pre-stimulation and levels were brought to similar levels as their mutant counterpart when PI3K activity was partially or completely inhibited (**Fig. 7A**). In contrast, PI(3,4,5)P3 levels showed no consistent evidence of inhibition by WT INPP4A (**Fig. 7B**), although assay results were highly variable. PI(3,4,5)P3 was measured at earlier time points as is tightly regulated and produced very rapidly and transiently after stimulation relative to

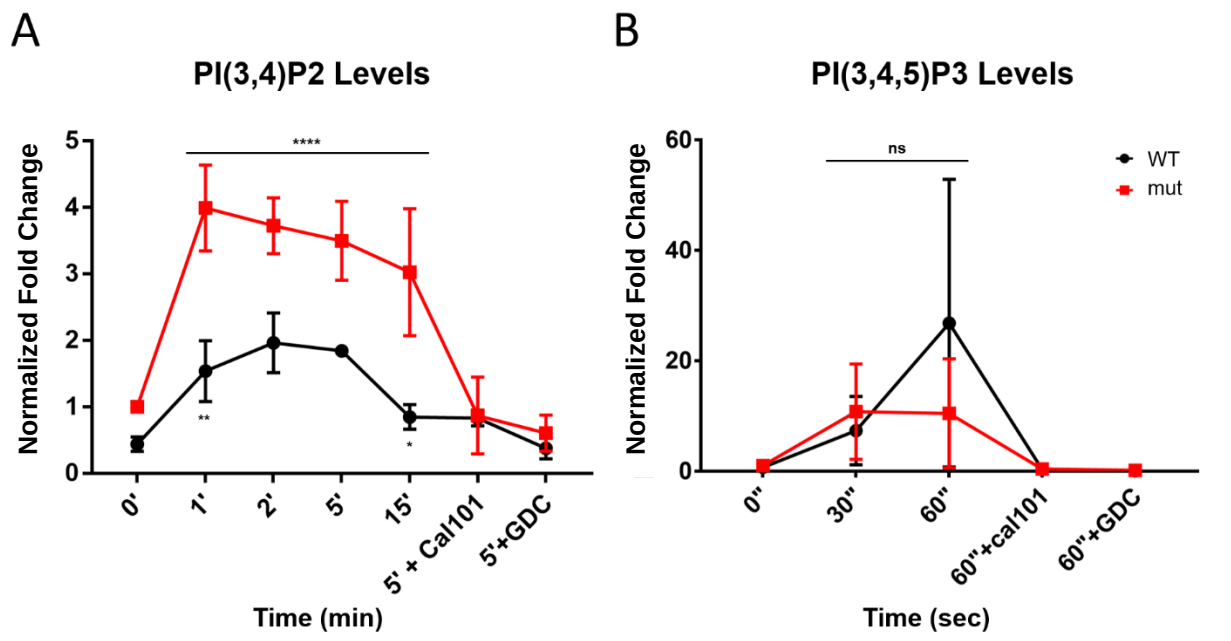


Figure 7. <u>PI(3,4)P2 levels reduced in transduced B cells overexpressing WT INPP4A during BCR stimulation and PI3K activity.</u>

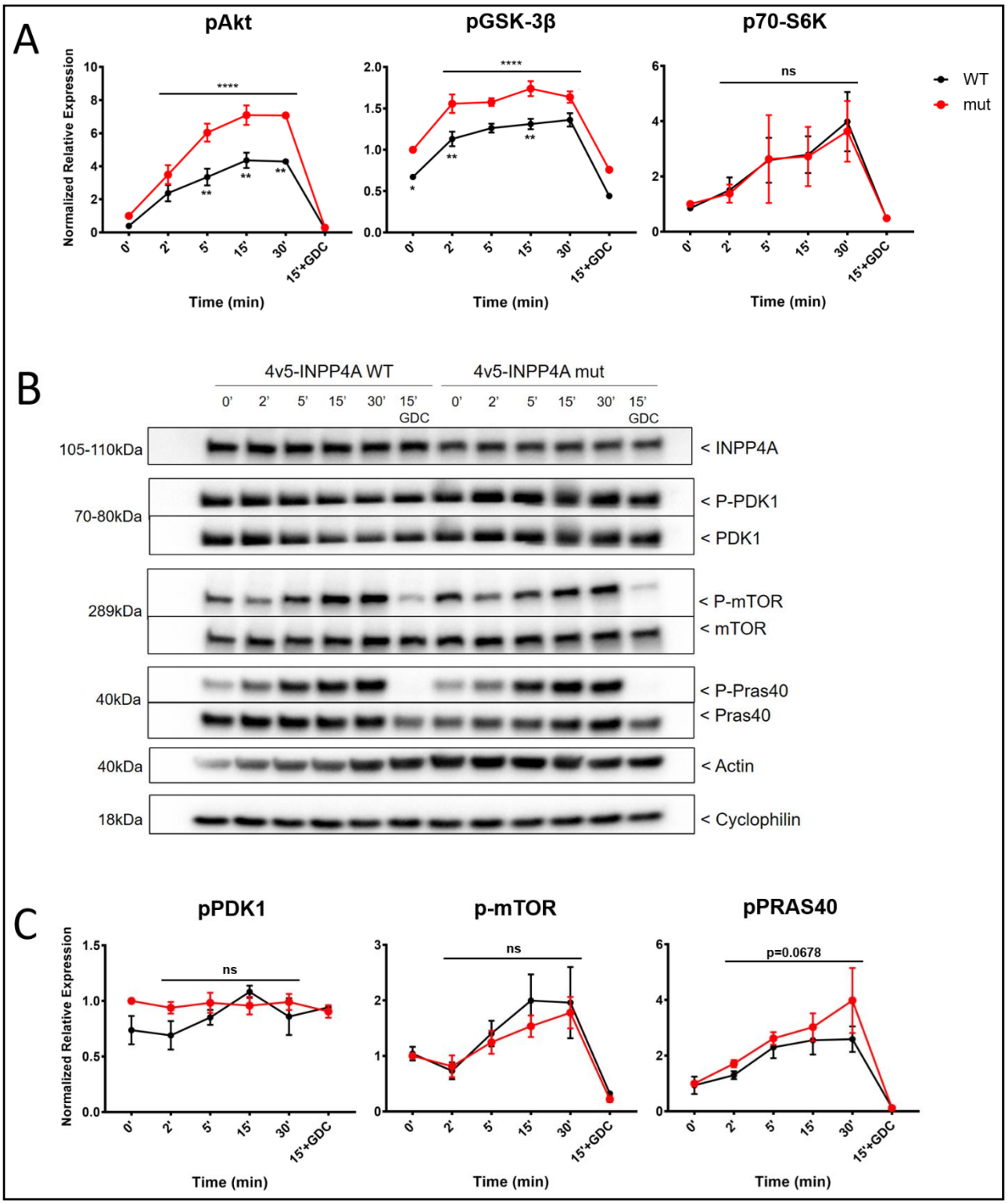
Competitive ELISA quantified the levels of PI's within stimulated 4v5-INPP4A and 4v5-INPP4A mut Bjab transductants at various time points. Pre-treatment with GDC-0980 (pan-PI3K inhibitor) or Cal101 (PI3Kδ inhibitor) acted as controls. All time points were stimulated with 2-10μg Goat Anti-Human IgM. PI(3,4,5)P3 and PI(3,4)P2 lipids were extracted and analysed. Lipid concentrations were obtained for each time point and then normalized to Bjab 4v5-INPP4A mut t=0min. **(A)** For PI(3,4)P2 analysis, Bjab cells were stimulated for 0min, 1min, 2min, 5min, 15min, 5min+Cal101, and 5min+GDC-0980. **(B)** For PI(3,4,5)P3 analysis, Bjab cells were stimulated for 0min, 0.5min, 1min, 1min+Cal101, and 1min+GDC-0980. All experiments were conducted to an n=3 and displayed as Mean with SEM. Statistical analysis was conducted with 2way ANOVA paired t-test for Time and Genotype (statistics shown for genotype). PI(3,4)P3 5min stimulation vs 5min+Cal101 and 5min stimulation vs 5min+GDC-0980 controls showed statistical significance (2way ANOVA Time comparisons p=0.0064 and p=0.0017, respectively); PI(3,4,5)P3 60sec stimulation vs 60sec+Cal101 and 60sec stimulation vs 60sec+GDC-0980 controls showed no statistical significance (2way ANOVA Time comparisons). $p \geq 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

PI(3,4)P2 [125, 126]. Overall, these results showed that cells overexpressing INPP4A WT significantly reduces PI(3,4)P2 levels relative to INPP4A mutants lacking phosphatase activity.

3.3 Reduced PI(3,4)P2 levels has negative effects on BCR-induced activation of Akt and some other downstream PI3K signaling proteins

My next step was to observe how reduced PI(3,4)P2 would affect downstream proteins of the PI3K signalling pathway. We focused on Akt since it is vital in cell metabolism, proliferation, and survival through its phosphorylation of downstream targets and is reported to require PI(3,4)P2 to be phosphorylated at S473 for full activation [58, 59]. Bjab 4v5-INPP4A WT and Bjab 4v5-INPP4A mut transduced cells were stimulated at various time points with 2µg/mL anti-human IgM. There was also an additional 15min time stimulation with cells pretreated with the pan-PI3K inhibitor GDC-0980 to determine whether protein phosphorylations of interest were PI3K-dependent or PI3K-independent (**Fig. 8**). Cell lysates were analysed using a mesoscale multiplex assay to detect pAkt (S473), total Akt, p70-S6K (T421/S424), total p70-S6K, pGSK-3β (S9), and total GSK-3β. The phospho/total protein ratios were normalized to the 0min 4v5-INPP4A mut relative expression value as it was considered the control. The normalized relative expression for Akt, p70-S6K, and GSK-3β were displayed (**Fig. 8A**).

In alignment with past results indicating that Akt requires both PI(3,4,5)P3 and PI(3,4)P2 levels to become fully phosphorylated [58, 59], overexpression of INPP4A WT inhibited Akt phosphorylation in Bjab 4v5-INPP4A WT compared to the 4v5-INPP4A mut cells (**Fig. 8A**). Overexpressing INPP4A WT also affected the downstream target GSK-3β (directly phosphorylated by Akt) as they saw significant decreased in phosphorylation levels (**Fig. 8A**). In contrast, p70-S6K showed no difference in phosphorylation levels between the 4v5-INPP4A WT and 4v5-INPP4A mut Bjab cells (**Fig. 8A**). Pre-treatment with the pan-PI3K inhibitor GDC-


A
pAkt
pGSK-3β
p70-S6K
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Time (min)
0'
2'
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*
ns
WT
mut
B
4v5-INPP4A WT
4v5-INPP4A mut
105-110kDa
< INPP4A
70-80kDa
< P-PDK1
< PDK1
289kDa
< P-mTOR
< mTOR
40kDa
< P-Pras40
< Pras40
40kDa
< Actin
18kDa
< Cyclophilin
C
pPDK1
p-mTOR
pPRAS40
p=0.0678

Figure 8. <u>Overexpression of INPP4A WT significantly decreased pAkt levels and the phosphorylation levels of some downstream targets during BCR stimulation.</u>

Transduced Bjab cells were stimulated with 2μg/mL F(ab')2 anti-human IgM at various time points. Cell lysates were quantified via mesoscale and western blot. Control cells were pre-treated with pan-PI3K inhibitor GDC-0980. **(A)** After stimulation, phosphorylated and total protein concentrations of Akt, p70-S6K, and GSK-3β were analysed by mesoscale. Protein lysates attach to specific antibody dots in the wells of the mesoscale plate for total Akt, pAkt (S473), total GSK-3β, pGSK-3β (S9), total p70-S6K, and p70-S6K (T421/S424). Relative phosphorylation was calculated (phosphorylated/total protein) and normalized to Bjab 4v5-INPP4A mut t=0min. **(B)** The same protein lysates were ran through western blot and incubated with separate primary antibodies for INPP4A and the following phosphorylated versions of the proteins: pPDK1 (S241), p-mTOR (S2448), and pPRAS40 (T246). Afterwards, membranes were stripped and re-incubated with their respective total protein primary antibodies (PDK1, mTOR, and PRAS40). Band intensities were quantified, relative phosphorylated expression calculated, normalized to Bjab 4v5-INPP4A mut t=0min, and displayed in **(C)**. n=3 for mesoscale and n=4 for western blot (Means with SEM), Statistical significance calculated by 2way ANOVA paired t-test for Time and Genotype (statistics shown for genotype). 15min stimulation vs 15min+GDC-0980 controls for Akt, GSK-3β, mTOR, and PRAS40 showed statistical significance (2way ANOVA Time comparisons $p=0.0019$, $p=0.0011$, $p=0.0045$, and $p=0.0011$, respectively); 15min stimulation vs 15min+GDC-0980 controls for 70-S6K and PDK1 showed no statistical significance (2way ANOVA Time comparisons). $p\geq0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

0980 prior to stimulation reduced phosphorylation of Akt, p70-S6K, and GSK-3β below their basal levels, confirming all three signaling proteins are PI3K-dependent.

Phosphorylation of downstream proteins PRAS40 and mTORC1, and the upstream Akt kinase PDK1 were also examined using western blot analysis. The same protein lysates collected for the mesoscale analysis were ran on gels, blotted and probed with antibodies for p-mTOR (S2448), pPRAS40 (T246), and pPDK1 (S241). Blots were developed, stripped, and then re-incubated with the primary antibodies of the non-phosphorylated forms of the proteins (**Fig. 8B**). I obtained the intensity values of the phosphorylated form and non-phosphorylated form of the same bands to calculate the relative expression for each protein (**Fig. 8C**).

The western blots validated that the transduced cells were expressing INPP4A WT and mut at high levels (**Fig. 8B**). Interestingly, while Akt showed a decrease in phosphorylation in the 4v5-INPP4A WT cells, the phosphorylation of upstream kinase PDK1 did not display a significant difference between the WT and mut Bjab transductants (**Fig. 8B and 8C**). While mTORC1 phosphorylation was significantly increased during stimulation in a PI3K-dependent manner, we surprisingly saw no inhibition of phosphorylation from INPP4A WT overexpression (**Fig. 8B**). The data quantified from the PRAS40 band intensities showed a trend of inhibited PRAS40 phosphorylation in Bjab cells overexpressing active INPP4A compared to the mut cells, however it did not reach statistical significance ($p=0.068$) (**Fig. 8C**). Analysis of mTORC1 and PRAS40 phosphorylation suggests PI3K-dependence as the GDC-0980 pre-treatment showed substantial decrease in relative phosphorylation, even compared to t=0min (**Fig. 8B and 8C**).

Together, these results have not only verified that depletion of PI(3,4)P2 from excess INPP4A activity caused a decrease in Akt phosphorylation, it also negatively affects the phosphorylation of some downstream targets such as GSK-3β and PRAS40.

3.4 Overexpression of active INPP4A does not significantly impact BCR-induced calcium flux or Btk/PLCγ activation

Phosphoinositides, more notably PI(3,4,5)P3, are important for the regulation of Btk/PLCγ leading to calcium release from the endoplasmic reticulum (ER) during BCR activation [60, 61]. In contrast, PI(3,4)P2 is not strongly linked to the influx of calcium during cell activation [20]. Btk and PLCγ phosphorylation was tested to observe if PI(3,4)P2 depletion by INPP4A may impact these events in an indirect way. Total Btk and phosphorylated Btk (Y223) were analyzed by western blots from cell lysates of 4v5-INPP4A WT and 4v5-INPP4A mut Bjab cells stimulated at various time points (**Fig. 9A**). Intensity values were collected from these western blots and normalized relative expression of Btk phosphorylation during cell stimulation was calculated and plotted (**Fig. 9B**). Overall, there was no difference in trend observed when comparing the overexpressing 4v5-INPP4A WT and 4v5-INPP4A mut phenotypes (**Fig. 9A and 9B**). Intracellular flow cytometry was used to examine the phosphorylation of PLCγ2 during cell stimulation. Intracellularly stained pPLCγ2 was quantified by flow cytometry showed no change in PLCγ2 phosphorylation between the 4v5-INPP4A WT and 4v5-INPP4A mut stimulated Bjab cells (**Fig. 9C**). As expected, pPLCγ2 was partially inhibited by PI3K inhibitors and strongly inhibited by a Syk tyrosine kinase inhibitor (R406) which shuts down BCR signaling.

Calcium assays were conducted to observe if calcium flux within the cells would be affected by INPP4A (**Fig. 7B and 9**). Cells were loaded with calcium sensitive dyes Fluo-4 and Fura-Red and analysed by flow cytometry (**Figure 10)**. Although there was variability between experiments, when taken all together, there was no apparent trend in internal calcium influx difference between Bjab 4v5-INPP4A WT and Bjab 4v5-INPP4A mut cells. This coincides with

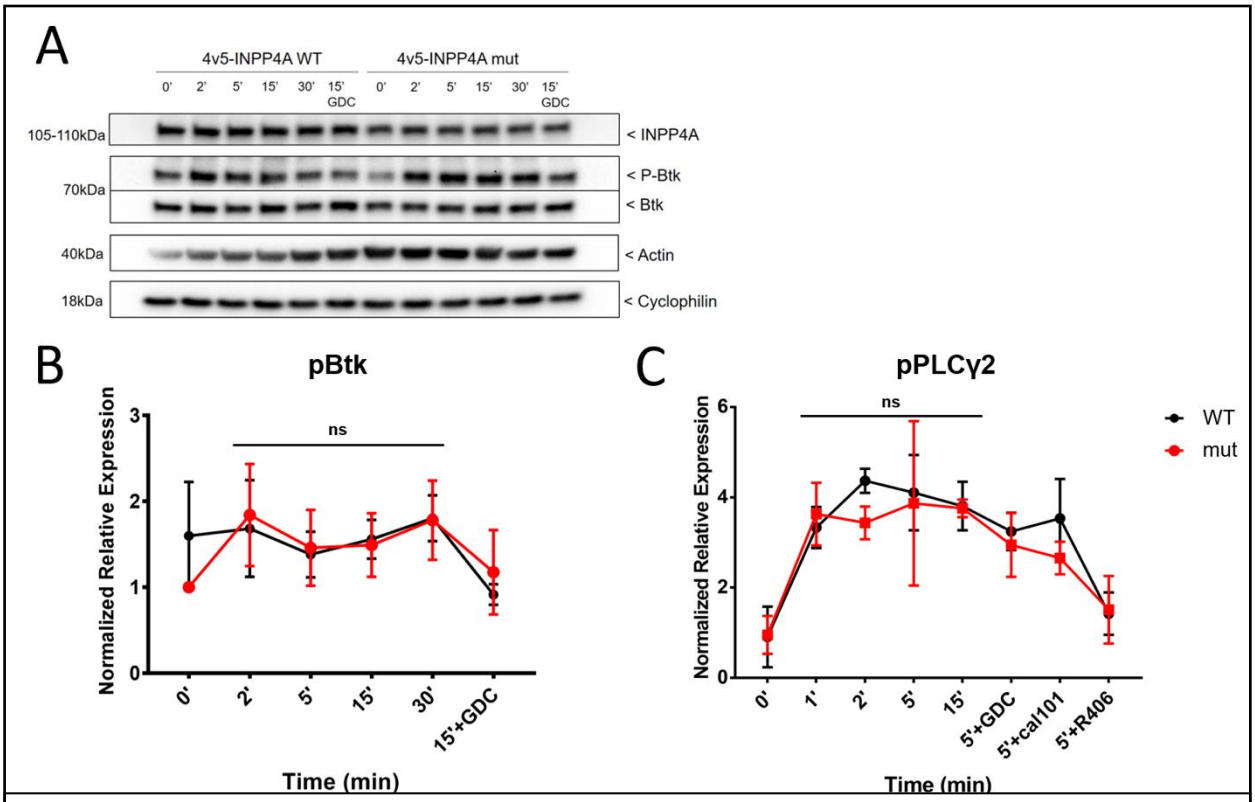

Figure 9. <u>Effector proteins Btk and PLCγ showed no difference in phosphorylation levels compared between B cells overexpressing INPP4A WT or INPP4A mut.</u>

(A) The cell protein lysates from a time stimulation assay was analysed by western blot incubated with primary antibodies for INPP4A and p-Btk (Y223). The Btk membrane was stripped and re-incubated with total Btk primary antibody. Band intensities were quantified, relative phosphorylated expression calculated, normalized to Bjab 4v5-INPP4A mut t=0min, and displayed in **(B)**. **(C)** Cells were stimulation with 10μg/ml goat F(ab')2 anti-human IgM for the respective time points. Control cells were pre-treated with GDC-0980 (pan-PI3K inhibitor), cal101 (PI3Kδ inhibitor), or R406 (Syk tyrosine kinase inhibitor). Cells were fixed, permeabilized, and immunostained intracellularly with Alexa Fluor® 647 Mouse anti-PLC-γ2 (pY759) and analysed by flow cytometry. Relative phosphorylated PLCγ2 expression was quantified (pPLCγ2/total PLCγ2) and values normalized to Bjab 4v5-INPP4A mut t=0min. n=2 for phospho-flow cytometry, n=4 for western blots, and displayed as Means with SEM. Statistical significance with 2way ANOVA paired t-test for Time and Genotype (statistics shown for genotype). 5min stimulation vs 5min+R406 controls showed statistical significance for PLCγ (2way ANOVA Time comparisons p=0.0312); 5min stimulation vs 5min+Cal101 and 5min stimulation vs 5min+GDC-0980 controls for PLCγ, and 5min stimulation vs 5min+GDC-0980 for Btk showed no statistical significance (2way ANOVA Time comparisons). $p \geq 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

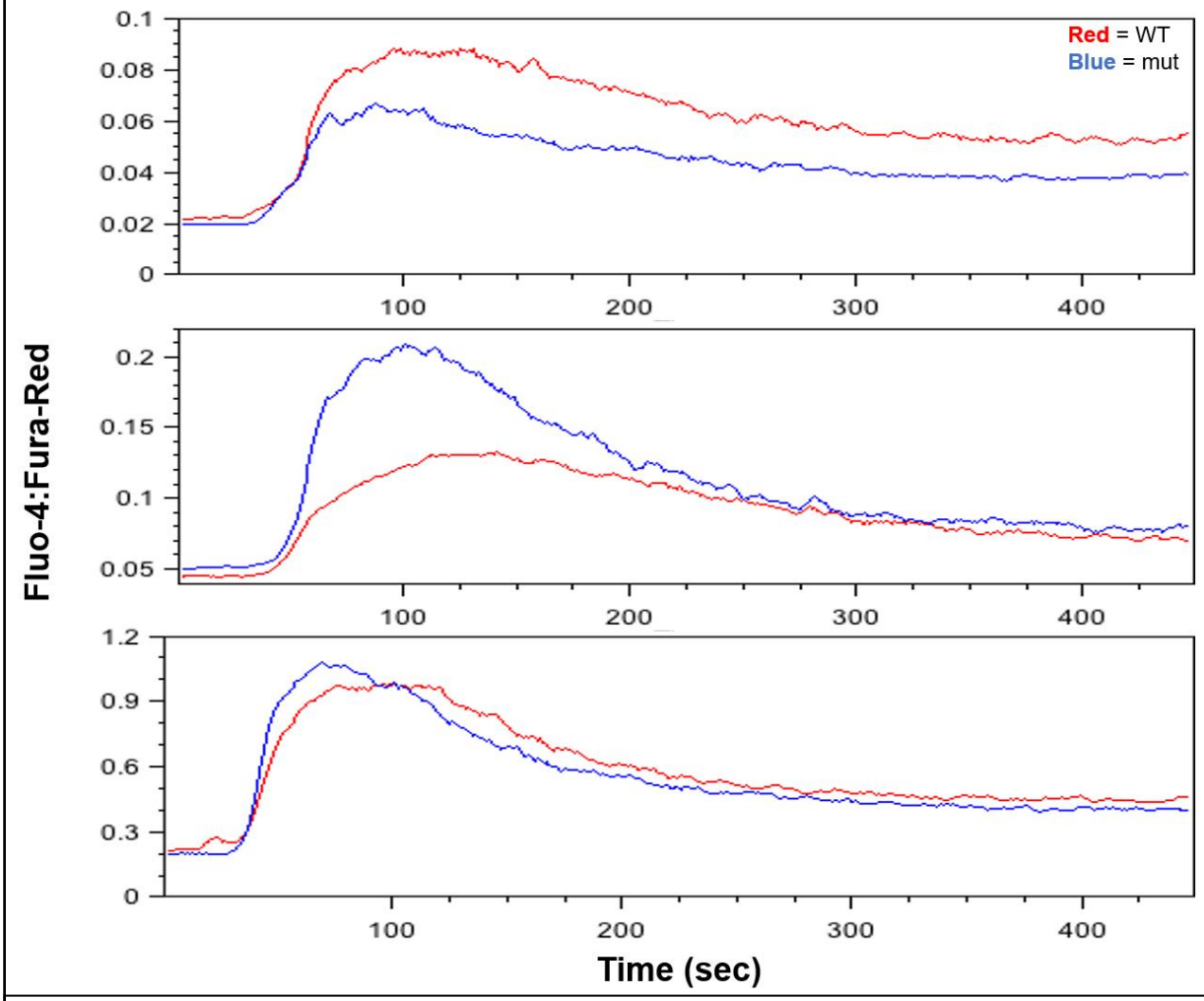

Figure 10. <u>No conclusive change in internal calcium influx within INPP4A WT transduced B cells during BCR stimulation compared to cells overexpressing INPP4A mut.</u>

Bjab 4v5-INPP4A WT and 4v5-INPP4A mut cells were intracellularly co-immunostained with Fluo-4 and Fura-Red calcium probes. Internal basal calcium levels were collected for 30sec from unstimulated cells by flow cytometry. Cells were then stimulated with 10µg/mL Goat Anti-Human IgM and calcium levels were detected for 7min. Results were merged with the basal calcium results for a time-kinetic assay. Flou-4 and Fura-Red were gated for Alex-649 and PerPC, respectively. The flux of internal calcium levels during BCR stimulation was quantified and displayed as a Flou-4:Fura Red ratio on a linear scale. Parameter were created by multiplying Fluo-4 (Alexa-647 fluorescent) by 10 and then dividing that by Fura Red (PerPC fluorescent) which was then all converted to 1:5 scale. The kinetic graphs were displayed using the Median values with moving average smoothing.

the findings of the relative phosphorylation of Btk/PLCγ which also indicated no difference in trend between Bjab 4v5-INPP4A WT and 4v5-INPP4A mut cells.

3.5 Akt recruitment to B cell plasma membrane was hindered in Bjab cells overexpressing active INPP4A

The next portion of this research was to observe the affects of recruitment of PH-domain containing proteins to the PM during BCR stimulation due to differing levels of PI's available. Specifically, the recruitment of Akt to the PM was tested to investigate if the decrease in Akt phosphorylation was due to decrease in Akt binding at the PM during BCR activation. To assess membrane association, I utilized confocal microscopy imaging of an Akt-EGFP fusion protein. Using electroporation, Akt-EGFP plasmid was transfected into Bjab 4v5-INPP4A WT, Bjab 4v5-INPP4A mut, and Bjab (control) cells. Transfected cells were stimulated with anti-human F(ab')2 IgM for 5min, with or without pan-PI3K inhibitor GDC-0980, washed, and then fixed in 2% PFA. Membrane recruitment of Akt-EGFP during BCR stimulation was observed using confocal microscopy and compared to cells that were left unstimulated or stimulated with GDC-0980 (**Fig. 11A**). Fluorescent intensities were quantified from the PM and cytoplasm of 25-30 cells per condition per cell line and average intensity ratios were calculated (PM/cytoplasm). The overall ratio was determined by taking the average value of all the individual ratios (**Fig. 11B**).

For all three cell lines, there was significant basal Akt-EGFP recruitment to the PM of unstimulated cells compared to the cells pre-treated with GDC-0980. This relatively high basal levels of Akt PM recruitment is likely due to Bjab cell being PTEN deficient [118]. As well, Bjab cells are Burkett's lymphoma cells (i.e. cancer cells) therefore are constantly proliferating and therefore basal levels of activated Akt is needed to maintain cell proliferation [127, 128].

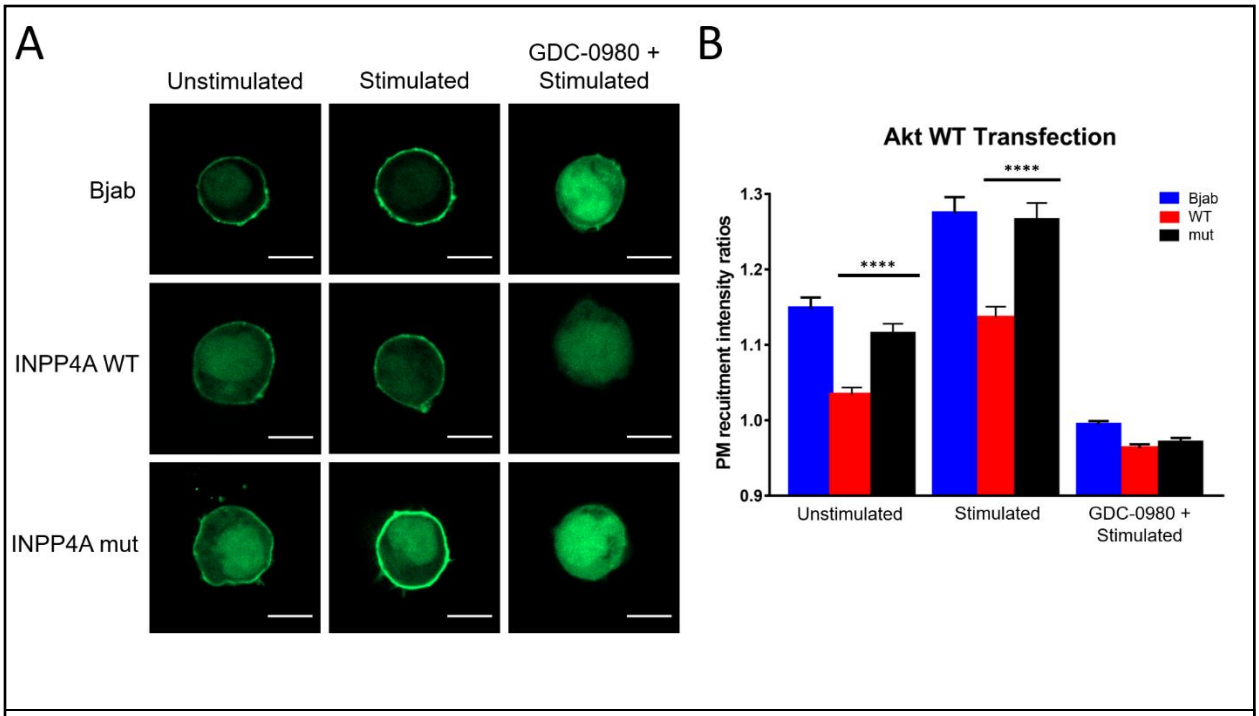

Figure 11. <u>Reduced Akt WT-EGFP plasma membrane recruitment observed in cells overexpressing INPP4A WT.</u>

WT Akt-EGFP tag plasmids were electroporated into non-transduced (parental) and 4v5-INPP4A WT and mut transduced Bjab cells. Control cells were pre-treated with the pan-PI3K inhibitor GDC-0980. Cells were stimulated for 5min with 10µg/mL anti-human $F(ab')_2$ IgM and fixed. **(A)** Akt membrane recruitment was observed and captured by confocal microscopy. PM recruitment intensity ratios were calculated by obtaining five mean fluorescent intensity values at the PM and the adjacent spots in the cytoplasm for each transfection conditions, averaging the fluorescents, and dividing the mean PM fluorescent intensity by the cytoplasm intensity (PM-intensity/cytoplasm intensity, this is the intensity ratio of an individual cell). Individual intensity ratios were calculated for 25-30 cells per condition per cell line. The overall PM recruitment intensity ratio displayed in **(B)** were obtained by averaging all the individual PM intensity ratios per condition. Nonparametric Mann-Whitney t-test determined the statistical significance of all condition comparisons. Statistical significance for Bjab 4v5-INPP4A WT unstimulated vs. Bjab 4v5-INPP4A mut unstimulated, and Bjab 4v5-INPP4A WT stimulated vs. Bjab 4v5-INPP4A mut stimulated were displayed. Representative cell images shown (n=3). $p \geq 0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

When compared to Bjab 4v5-INPP4A mut, Bjab 4v5-INPP4A WT showed a significant decrease in Akt-EGFP membrane recruitment whether stimulated or not stimulated (**Fig. 11B**). For all cell lines, GDC-0980 severely depleted Akt-EGFP recruitment to the PM during BCR stimulation (**Fig. 11**). Furthermore, when comparing Bjab parental to Bjab 4v5-INPP4A mut cells in both unstimulated and stimulated conditions, it was observed and quantified that they shared similar levels of Akt-EGFP PM recruitment. The results gathered here coincide with the previous pAkt mesoscale results (**Fig. 8A**) and it could be concluded that the decrease in Akt phosphorylation during BCR stimulation is possibly due to the decrease of physical Akt membrane recruitment, which in turn is most likely due to the depletion of available PI(3,4)P2.

3.6 Impact of INPP4A overexpression on glycolytic activity

Glycolysis is a common indicator of elevated metabolic activity in cancerous cells and is known to regulated by the PI3K pathway. Hence, glycolysis was measured to observe the affects of active INPP4A overexpression at the cellular level. To measure glucose metabolism in these cells during stimulation, extracellular acidification rate (ECAR) measurements were collected. The changes in extracellular media pH measured are caused by the release of free protons during the conversion of glucose to pyruvate to finally lactate, and thus allows for a direct real-time measurement of glycolysis. The assay protocol was modified to observe the glycolytic activity of cells overexpressing functional or catalytically dead INPP4A during cell stimulation and/or PI3K inhibitor treatment. The five assay steps were designed as followed: 1) collection of basal ECAR levels, 2) treatment with either media or the pan-PI3K inhibitor GDC-0980, 3) stimulation with anti-human f(ab')$_2$ IgM or a blank, 4) addition of glucose to measure glycolysis levels, and 5) 2-DG introduced to shut down glycolysis and end the experiment. This

modification closely resembles the steps prior and during stimulation of the other experiments that were conducted to ensure consistency.

Bjab 4v5-INPP4A WT and 4v5-INPP4A mut cells were compared with or without anti-IgM stimulation to observe how the overexpression of INPP4A affects glycolysis under each of these conditions. Anti-IgM stimulation appeared to result in an increase in glycolytic activity (although statistically not significant) in 4v5-INPP4A mut Bjab cells, while their WT counterparts showed no increase after stimulation (**Fig. 12A**). The average basal ECAR levels prior to cell stimulation and glucose addition was significantly reduced in 4v5-INPP4A WT Bjab cells (**Fig. 12B**). This hints at an inhibitory role of overexpressed active INPP4A on glycolysis in these cells prior to stimulation conditions. 4v5-INPP4A WT and 4v5-INPP4A mut Bjab cells were stimulated for approximately 30min or left unstimulated and then glucose was added to compare the average glycolytic rate between the two cells lines. What was shown was Bjab 4v5-INPP4A WT cells had an decreased trend in average glycolytic activity than the mutant cells which was more significant after cell stimulation (**Fig. 12C and 12D**).

To confirm that the glycolysis measurements collected were PI3K-dependent, we compared glycolytic activity of Bjab 4v5-INPP4A WT (**Fig. 13A**) and mut cells (**Fig. 13B**) that were pre-treated with media versus GDC-0980 prior to cell stimulation with IgM and addition of glucose. It was found that 4v5-INPP4A mut Bjab cells showed a significant reduction in the average glycolytic activity when pre-treated with GDC-0980 (**Fig. 13C and 13D**). In contrast, 4v5-INPP4A WT cells showed no significant reduction with GDC-0980 treatment (**Fig. 13A and 13B**). This may be due to the overexpression of active INPP4A inhibiting glucose metabolism to an extent that the shut down of PI3K activity does not inhibit glycolysis that much further.

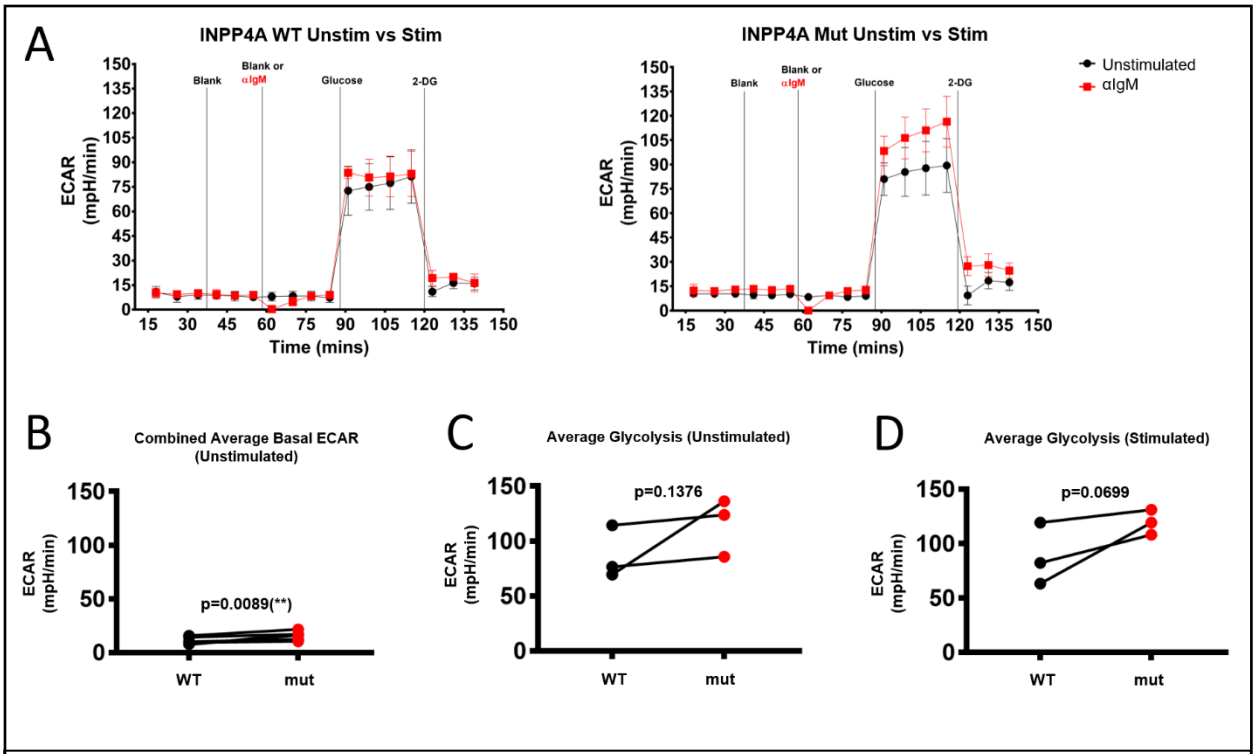

Figure 12. <u>Glycolytic activity is negatively affected in B cells overexpressing INPP4A WT in stimulation conditions.</u>

XF sensor cartridge was prepared to inject solutions to cells: <u>INJECTION A</u> – media blank as a pre-treatment, <u>INJECTION B</u> – 10µg/mL anti-human F(ab')$_2$ IgM or a media blank for stimulated or unstimulated conditions, <u>INJECTION C</u> – 5mM D-glucose solution to begin glycolysis, and <u>INJECTION D</u> – 20mM 2-Deoxy-D-glucose solution (2-DG) to shut down glycolysis. The rate of glycolysis was outputted as ECAR through changes in pH levels, and displayed in **(A)** showing differences in glycolytic rates when cells were either stimulated or unstimulated for Bjab 4v5-INPP4A WT and 4v5-INPP4A mut cells. Representative graphs are shown. **(B)** Average basal ECAR measurements were calculated from the three time points prior to Injection A from samples that were left unstimulated. Basal ECAR values from Figures 13A (Bjab 4v5-INPP4A WT) and 13C (Bjab 4v5-INPP4A mut) with no drug pre-treatment were also included for experimental replicates n=6. Glycolytic ECAR measurements (immediately after D-glucose Injection C) were calculated from the four measurement points comparing Bjab 4v5-INPP4A WT and Bjab 4v5-INPP4A mut samples in unstimulated **(C)** and stimulated **(D)** conditions. Each point represents a separate experimental replicate (n=3). Statistical significance was tested using parametric paired t-tests. $p \geq 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

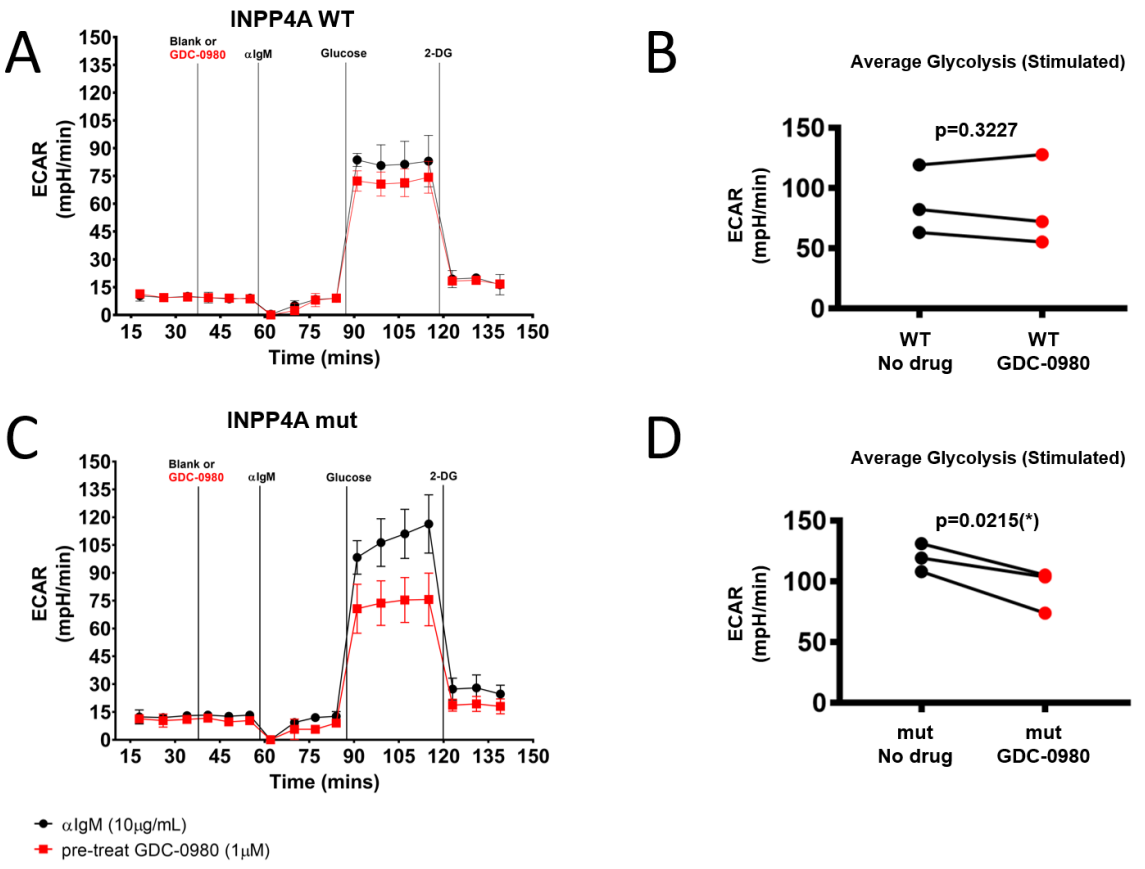

Figure 13. <u>PI3K inhibitor pre-treatment did not cause further inhibition of glycolysis in INPP4A WT B cells compared to no drug treatment during BCR stimulation.</u>

XF sensor cartridge was prepared to inject solutions to cells: <u>INJECTION A</u> – 1μM pan-PI3K inhibitor GDC-0980 or a media blank as a pre-treatment, <u>INJECTION B</u> – 10μg/mL anti-human $F(ab')_2$ IgM for stimulation conditions, <u>INJECTION C</u> – 5mM D-glucose solution to begin glycolysis, and <u>INJECTION D</u> – 20mM 2-Deoxy-D-glucose solution (2-DG) to shut down glycolysis. The rate of glycolysis was outputted as ECAR through changes in pH levels, and displayed in **(A)** and **(C)** showing the impacts on glycolysis when cells were pre-treated with GDC-0980 for Bjab 4v5-INPP4A WT and 4v5-INPP4A mut cells, respectively. Representative graphs were shown. Average glycolytic ECAR measurements (immediately after D-glucose Injection C) were calculated from the four measurement points within the time range in treated Bjab 4v5-INPP4A WT and Bjab 4v5-INPP4A mut cells and displayed in **(B)** and **(D)**, respectively. Each point represents a separate experimental replicate (n=3). Statistical significance was tested using parametric paired t-tests. $p \geq 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Chapter 4: DISCUSSION

INPP4 is a vital negative regulator of the PI3K signaling pathway along with the two well studied phosphatases PTEN and SHIP [41, 43]. While there are studies examining the roles and consequences of PTEN and SHIP in B cells, no studies have examined how INPP4 aids in the regulation of BCR signaling in B cells. In this study, the goals were to understand how PI(3,4)P2 hydrolysis would affect the two main downstream branches of the PI3K signaling pathway and the impact on B cell metabolic activity.

The results indicate that INPP4A overexpression significantly reduces PI(3,4)P2 levels whereas PI(3,4,5)P3 showed no reproducible changes. Reproducibly issues with PI(3,4,5)P3 analysis was mostly likely due to PI(3,4,5)P3 levels rising, peaking, and quickly declining within a span of a minute, whereas PI(3,4)P2 will peak at approximately 60sec, plateau, and then slowly decrease in the span of 30min [20, 36, 59]. The procedure to obtain the PI lipids from cells after BCR stimulation is lengthy and intricate so it was possible the brief time frame of when PI(3,4,5)P3 are still elevated in these cells was missed, even when there were two people performing the extraction process to minimize the gap between the time of stimulation to lipid extraction. The HPLC-Mass Spectrometry approach depicted in Li *et al*. (2015) and Malek *et al*. (2017) may have been more accurate [67, 69]. Nevertheless, our results are still believed to hold relevance by clearly demonstrating the effect of INPP4 on PI(3,4)P2, although the lack of effect on PI(3,4,5)P3 is less certain at this point. From these results, experiments on the Akt and the Btk/PLCγ branches and their downstream targets in the PI3K signaling pathway were conducted.

Overexpression of INPP4A resulted in a clear decrease in Akt phosphorylation and subsequent decrease of some, but not all, of its downstream targets. Akt must be phosphorylated at positions T308 and S473 to become fully activated and they are respectively phosphorylated

by PDK1 and mTORC2 [76, 78, 79]. Our assay examined the S473 phosphorylation site which is phosphorylated by mTORC2. Therefore, Akt may still be phosphorylated in its active loop at T308 by PDK1 which primes Akt to be phosphorylated at S473 by mTORC2 for full activation [29, 74]. However, based on our results and literature on PDK1 activity, we would predict that PDK1 activity is not affected by INPP4A. For PDK1 to become activated, it strictly binds to available PI(3,4,5)P3 at the PM in order to auto-phosphorylate the S241 position within its active domain to reverse to inhibition on its kinase activity [73, 75]. As we found no reproducible trend in PI(3,4,5)P3 depletion or altered PDK1 phosphorylation in WT INPP4A expressing cells, it seems likely that activation of PDK1 is unaffected.

As for the S473 site of Akt, the significant decrease in phosphorylation could be explained as Akt requires both PI(3,4,5)P3 and PI(3,4)P2 to bind onto and become activated. Early studies on Akt show it binds PI(3,4)P2 with a higher affinity than PI(3,4,5)P3 [40, 59]. My confocal microscopy data showed a significant inhibition of Akt recruitment to the PM during cell stimulation as well as a significant decrease in basal Akt recruitment in Bjab 4v5-INPP4A WT cells. These results suggest decreased Akt S473 phosphorylation is related to reduced membrane association. The impaired membrane association might hinder the ability of mTORC2 to phosphorylate S473. This is consistent with a previous study showing that PI(3,4)P2 is more important for S473 phosphorylation whereas PI(3,4,5)P3 is more important for T308 phosphorylation [117].

This decrease in Akt S473 phosphorylation also seems to translate to a decreasing trend in glycolysis in Bjab cells overexpressing active INPP4A. Akt is important in glucose metabolism through the induction of HK2 and regulating GSK-3 [26, 72]. We found reduced GSK-3β

phosphorylation at S9 Bjab cells overexpressing active INPP4A. Phosphorylation of GSK-3β at S9 by Akt inhibits its activity therefore a lack of phosphorylation would presumably lead to the prolonged activity of GSK-3β and decrease metabolic processes [129]. GSK-3 has been reported to have between 50-100 substrates and is active in quiescence cellular states and inhibited once phosphorylated by Akt or other kinases [95, 97]. In its active state, GSK-3 inhibits downstream metabolic pathway in B cells including glycolysis by phosphorylating the proto-oncogene cMyc which is involved in cell proliferation and common in cancer such as Burkitt's lymphomas [26, 130]. Conversely, knockout of GSK-3 induced an increase in ECAR and OCR in B cells [131]. As it was shown through the ECAR assays done here, decrease in GSK-3β phosphorylation has a net negative effect on metabolism in cells overexpressing WT INPP4A and decreased Akt phosphorylation.

Not all downstream targets of Akt were negatively affected in Bjab cells overexpressing INPP4A WT during BCR-ligation and PI3K activity. Western blot and mesoscale analysis on mTORC1 and p70-S6K relative phosphorylation expression during B cell stimulation showed no significant inhibition in cells overexpressing INPP4A. According to Copp *et al*. (2009), Akt phosphorylates the S2448 site of mTORC1 within its Raptor domain which then allows mTORC1 to activate p70-S6K [79]. It has been shown that when Akt is hyperactivated there is a hyperactivity of mTORC1, usually from an increase in PI(3,4)P2 levels from dysregulation of the upstream PI3K phosphatase regulators like PTEN or INPP4 [82]. However, the results here show that the decrease in Akt phosphorylation and activity did not inhibit mTORC1 phosphorylation. This may indicate that residual Akt activity in INPP4A WT cells is sufficient to activate mTORC1 or that other kinases can activate this. Our results suggest that GSK-3β is more sensitive to impaired PI(3,4)P2-dependant activation of Akt than is mTORC1. The reason

that mTOR activation is less dependent on Akt could be that it receives input from multiple signaling pathways including mitogen-responsive pathways, energy-sensing pathways, response to hypoxia (1% O_2 conditions), regulation by amino acids, and regulation by phospholipase D and phosphatidic acid [132].

p70-S6K was also examined even though it is not a direct target of Akt activity through phosphorylation, but rather an indirect target of Akt activity via its phosphorylation by mTORC1. p70-S6K is phosphorylated at T389 by mTORC1, though the phosphorylation sites we assayed were T421 and S424. This is because these sites, along with S411 and S418, must be phosphorylated to promote T389 phosphorylation by mTORC1 (**Fig. 5**) [84-86]. p70-S6K regulates protein synthesis through the ribosomal subunit S6 substrate and inhibiting pro-apoptotic molecules. If p70-S6K activity was compromised (i.e. if its activity was decreased due to decreased Akt activity from increased INPP4A activity) it could have severely compromised cellular proliferation processes and protein synthesis [83, 84]. Therefore, more severe consequences than just a decrease trend in glycolysis in ECAR assays could hypothetically have been detected.

The other vital branch of the PI3K pathway is the Btk/PLCγ calcium pathway. Although INPP4 is not associated with PI(3,4,5)P3 directly, which is known to be critical for Btk/PLCγ activation, we wanted to see if INPP4 might indirectly affect this pathway. One potential mechanism could be to alter the production of PI(3,4,5)P3 to accommodate the excessive hydrolysis of PI(3,4)P2, thus affecting the downstream targets of PI(3,4,5)P3 [20, 57]. Another possibility was that reduced recruitment of PI(3,4)P2 binding proteins might indirectly affect the Btk/PLCγ calcium pathway. For example, TAPP1/2 is a PH-domain containing protein abundant in immune cells that strictly binds to PI(3,4)P2 and promotes the recruitment of the inhibitory

phosphatase PTPL-1 [20, 105]. However, western blot and phospho-flow cytometry analysis revealed no significant differences in the relative phosphorylation expression of Btk and PLCγ or calcium flux between Bjab 4v5-INPP4A WT and 4v5-INPP4A mut cells.

INPP4A WT is predicted to globally decrease membrane recruitment of PI(3,4)P2 binding proteins without impacting PI(3,4,5)P3-binding proteins. I attempted to visualize the PI levels within these B cells during BCR activation and determine the effects of overactive INPP4A WT on PM recruitment of Btk (PI(3,4,5)P3-specific) and TAPP2 (PI(3,4)P2-specific) using confocal microscopy. It would be expected to see no difference in PM recruitment and binding for Btk-EGFP, whereas for TAPP2-mCherry it would be expected to see a decrease in PM recruitment much like Akt-EGFP. Confocal microscopy experiments were attempted but there were issues when these plasmids were either co-transfected or transfected individually into INPP4A expressing cells. PM recruitment was not being observed in any conditions whether it be unstimulated, 30sec, 1min, or 5min stimulation, or pre-treated with GDC (data not shown). A possible reason for the lack of observed Btk PM recruitment could be a technical issue with the cell fixing protocol using 2% PFA, since PI(3,4,5)P3 tend to rapidly increase and then decrease in the span of a minute thus Btk binding may be missed. A way to rectify this would be to do live cell imaging with confocal microscopy. As for the TAPP2 transfection, more optimization trials need to be conducted and possibly live cell imaging may need to be utilized as well.

Many of the previous studies on INPP4 looked at its effects in disease and cancer models in various cellular contexts, however not in B lymphocytes. With the early characterization of INPP4's role as a tumor suppressor in various cancers, many studies of INPP4 has revolved around understanding what happens when INPP4 is downregulated. Typically, these studies detailed the same thing; the decrease in INPP4 expression and activity, particularly INPP4B,

resulted in Akt hyperactivation and tumor growth due to the increase in PI(3,4)P2 levels within various cancer cell models. These cancer models include breast cancer, epithelial cancer, ovarian cancer, prostate cancer, thyroid and endometrial cancers, melanoma, malignant proerythroblast cells, osteosarcoma cells, and most recently lung cancer, and bladder cancer to name a few [37, 41, 52, 53, 82, 116]. Increase in PI(3,4)P2 levels and hyperactivation of Akt from downregulated or knocked out INPP4 expression was also observed in other cellular contexts leading to asthmatic inflammation, increased phagocytosis in macrophages, and the impairment of pro-inflammatory cytokine function and bacterial clearance by myeloid cells for example [41, 46, 56, 112].

Our research is very distinct in that INPP4A function was examined via overexpression in B lymphocytes. When INPP4 is overexpressed, its role in disease or cancer pathology has been particularly shown to depend on the cellular context. In some cancer models, for example human mammary tumor cell lines and hepatocellular carcinomas, increased INPP4B surprisingly resulted in decreased tumor growth and migration due to the decrease in Akt phosphorylation [52, 114]. In other words, when overexpressed INPP4B showed the opposite effects and acts as a tumor suppressor. However, one published study has cast doubt on the specificity of INPP4B for PI(3,4)P2, potentially explaining why it has been found associated with cancer, whereas INPP4A has not [37]. To our knowledge, overexpression studies on INPP4A have not been carried out in any cell types. Our results parallel those that were depict in this research as the increase in INPP4A function lead to the decrease in PI(3,4)P3 levels and therefore a decrease in Akt activation. They also showed similar results to the migratory research done in the past on other B cell lines (Raji) and CLL primary cell lines [69]. However, none of these studies looked at the effects on downstream signaling targets of PI(3,4)P2 and Akt.

As mentioned in the Introduction chapter, INPP4B is an oncogenic driver when overexpressed. In some cases, this oncogenic role of INPP4B tends to lead to poor outcomes and therapy resistance in cancer patients and thus low survivability [111]. The effects of INPP4 overexpression have been shown to have consequences dependent or independent of PI-phosphatase function. For example, in AML patients an increase in INPP4B function decreases PI(3,4)P2 levels however there was no connecting effects to endocytic processes or reduction in Akt phosphorylation at position S473. Therefore, INPP4B seems to have a different role in myeloid leukemia [55]. Of course, this was different than what was observed here when overexpressing INPP4A in the Burkitt's lymphoma Bjab cell lines where a decrease in S473 pAkt was observed due to the decrease in PI(3,4)P2 levels during BCR activation. In other cell lines, increase in INPP4B saw no affects on Akt phosphorylation during cell stimulation or even affects on basal levels during quiescence in melanomas, indicating an Akt independence through PI3K signaling [54].

Most of the studies that were described here that did look at overexpression of INPP4 only looked at the affects to Akt and occasionally PI levels. Only a couple went further and observed the effects overexpressing of functional INPP4 could have on signaling targets downstream of PI(3,4)P2 and/or Akt. Recently, a study on the overexpression of INPP4B in colorectal cancer cells looked at the effects of Akt and mTORC1 phosphorylation and what they recorded was a surprising increase in Akt and SGK3 activity leading to the increase in mTORC1 activity [82]. While the study did look at a downstream target of Akt, the results did not parallel to the results gathered here with a decrease in Akt phosphorylation and a trend of no change between overexpressed INPP4A WT and INPP4A mut in transduced Bjab cells. A major factor that could

be showing these differences is the simple fact that the observations were done in two completely different cell types.

4.1 Strengths, limitations, and significance of this research

The B cell model utilized in this Thesis, proved to be useful to assess biochemical differences when INPP4A is purposely overexpressed, yet the cellular effects were almost nominal. As mentioned, Bjab cells are a Burkitt lymphoma B cell cancer line with a PTEN deficiency [118]. Therefore, one limitation of the study is that there is a possibility of PTEN-related genomic instability and/or overall cancer cell adaptability within this system leading to no noticeable anti-cancer effect of INPP4A overexpression, despite significant biochemical effects which would have either highly positive of negative effects in a normal/non-cancerous cell. With a PTEN deficiency, B cells would display pro-oncogenic characteristics including hyper-proliferation, resistance to apoptotic stimulation, and an increase in Akt activity [133]. Thus while overexpression of INPP4A in this system has the advantage of displaying biochemical effects, cellular effects may be masked or muted as immortalized cancer cells are also highly adaptable in order to survive their internal and external environments [134]. In a non-transformed primary tumor cell setting it is theorized that an overexpression of INPP4A could have a much greater cellular effect. Which is why an alternative model, for example an INPP4A inducible model, would be beneficial.

A tetracycline (Tet)-controlled system is an example of a gene inducing system that gives the experimenters complete control of when a protein of interest is expressed within cells and is reversable. Doxycycline (Dox) acts as the inducer in order to either activate or inactivate transcription [135]. Using this model, INPP4A overexpression can be turned off and on again and both biochemical and cellular effect would then be observed. This would help eliminate the

limitation of the current stable-transduction system with INPP4A being continuously overexpressed may adapt to the overexpression over time. In other words, my cellular model used in this Thesis gives a biochemical insight and the inducible model proposed would be more likely to reveal give the functional effect on cells. The biochemical results that were shown here would be able move this research forward into that direction as this is the first time the role of INPP4A in BCR activation and B cell metabolism were researched and now we can take it one step further into translational research.

The significance of this research is it is among the few current studies that describes the distinct effects of the signaling targets just downstream of PI(3,4,5)P3 and PI(3,4)P2, rather than the effects of PI3K activity as a whole. Moreover, it is the first research to be conducted looking at the role of INPP4A in BCR activation and B cell metabolism. This is important as defects in B cell activity, whether it is upregulated or downregulated, can cause serious negative effects because of its role in the adaptive immune system and general immunosurveillance in the host. These defects can stem from dysregulation of the tightly controlled downstream signaling pathways. Understanding the mechanisms of these signaling pathways and their dysfunction in disease has led to current therapies against cancer and autoimmune diseases [40, 45, 136]. Though it has been studied in other cellular contexts, this study is the first to demonstrate that INPP4 is also an important phosphatase that can regulate the PI3K signaling pathway activated during BCR ligation. These results could further lead the general research in understanding B cells and the early stages of activation. Even currently, the promising results gathering from the *in vitro* studies conducted here have led to new *in vivo* mouse models being created.

If my results observed in *in vitro* in an immortalized cancer cell line were to parallel future *in vivo* results in primary cells, this could possibly lead to therapeutic targeting of INPP4A either

individually or in combination with other drugs, for example SHIP activators. Small molecule activators for INPP4A could be a potential future therapeutic development as the model used in this study strongly suggests active INPP4A can cause significant biochemical changes within B lymphoma cells. One limitation to therapeutic development is our current lack of knowledge about the protein expression levels of INPP4A/B in normal B cell subsets and in different types of cancerous B cells. Cancer cells have been shown to change their expression levels of a particular protein, which could be a possible explanation of why endogenous levels of INPP4A appear to be low in parental non-transduced Bjab cells [137]. However, more research needs to be conducted on the endogenous expression of INPP4A/B in lymphocytes moving forward.

4.2 Future research

The experiments conducted and results displayed here are an excellent start in depicting the convincing story of the importance on INPP4A and its effects on PI levels, downstream target activation in BCR activation, and B cell metabolism. Unquestionably, research is a continuous progress and while improvements on current experiments have been suggested, future experiments stemming from these experiments would benefit in understanding the function of INPP4 in BCR activity further.

Using Seahorse ECAR assays, it was tested if the glycolytic measurements gathered were PI3K-dependent. A future experiment would be to pre-treat the cells with the allosteric Akt inhibitor MK-2206. This will suppress Akt phosphorylation and is currently used in some cancer treatments [138]. This would determine whether the differences in glycolytic rates that were observed during BCR stimulation are Akt-dependent. Akt is vital in cellular metabolism as it regulates the signaling proteins associated with this process [26, 72]. However, from an evolutionary standpoint it would be irresponsible of a cell to make Akt the only point of

initiation for cell metabolism, especially glycolysis. The downstream target mTORC1 is involved in glucose metabolism and as mentioned prior mTOR can receive Akt-independent signaling for activation based on the no changes in mTORC1 phosphorylation western blots in Bjab 4v5-INPP4A WT cells compared to Bjab 4v5-INPP4A mut cells [77, 132].

These transduced cell lines still have endogenous INPP4 genes intact therefore these cells can still produce functioning INPP4A; however, we have found that Bjab cells have very low endogenous expression of INPP4. Therefore, the Bjab 4v5-INPP4A mut cells were treated as our control group to determine phosphatase-dependent activities of overexpressed INPP4A. To determine whether INPP4A overexpression may have phosphatase-independent effects as well, we need to reconduct the major experiments comparing INPP4A mut cells with an empty vector B cell control. These Bjab-Empty B cell lines will have gone through the transduction process like the Bjab 4v5-INPP4A cell lines but with the only difference being there is no overexpression of active or catalytically dead INPP4A. Since INPP4A mut showed similar Akt membrane recruitment as parental Bjab cells, we expect that the Bjab-Empty vector controls will show similar results to the parental and 4v5-INPP4A mut Bjab cell lines regarding Akt activation.

Bjab cells are a Burkitt-like lymphoma cell [127, 128]. In the past, our group has conducted studies on Raji cells, which are also Burkitt's B lymphoma cells, when observing the effects on B cell migration when WT INPP4A was overexpressed [69]. Although Raji cells express low levels of BCR and are a less preferred model for BCR signaling studies, nevertheless the next step is to reconduct key experiments in Raji B cells lines with intact PTEN to confirm the finding from Bjab cells before conducting experiments in an *in vivo* mouse system. This would ensure that the results gathered were not the result of the Bjab cell line's characteristic but rather they are events that would occur in B cells in general when INPP4A is dysregulated. It is

hypothesized that the results from Raji cells overexpressing active or inactive INPP4A will be similar to Bjab even though there will be less PI(3,4)P2 available due to the function of PTEN.

Previous studies found that INPP4A colocalizes to early and recycled endosomes both in quiescent and stimulated cell states [109, 139]. This colocalization to the early endosome hydrolyses the available pool of PI(3,4)P2 to PI(3)P [41, 109, 110]. Recently, the production of PI(3)P, in combination with PI(4,5)P2, has been investigated to recruit SNX9 (a protein involved in endocytosis and vesical trafficking) and activate actin assembly needed for endocytosis [140]. To prevent overactivation of B cells, BCR signaling must be downregulated by endocytosis. In B cells, the function of endocytosis is two-fold; first, it downregulates BCR signaling by BCR internalization and secondly, to uptake antigens that have bound to the BCR for processing to be placed on MHC II molecules on the B cell surface to present to CD4+ Helper T cells [141-144]. Thus, its possible that INPP4A may impact B cell activation via effects on endocytosis. To test this, flow cytometry and confocal microscopy assays could be utilized to measure the rate of BCR endocytosis after stimulation in INPP4A overexpressing cells by transfecting B cells with the EGFP endosome probes (ex. SNX9) and incubating cells with labelled anti-human $F(ab')_2$ IgM [109, 142, 145]. Conducting these experiments would provide a more complete story of the overall role INPP4 has on BCR activity and its downstream signaling as well as regulating its overall shutdown. It is hypothesized that with the increase production of PI(3)P from the overactivity of overexpressed INPP4A, it would facilitate an increase recruitment of SNX9, and therefore an increase in endocytosis and B cell shutdown through BCR internalization [140].

Finally, to observe the effects overexpression of functioning INPP4A would have on normal, non-lymphoma B cell signaling and metabolism, experiments will need to be conducted in an *in vivo* system. Currently, a mouse model is being created to specifically overexpress INPP4A in B

cells. To induce B cell specific overexpression of WT INPP4A, a Cre-Lox system, known as a Lox-STOP-Lox (LSL), was used to create conditional transgenic mice [146]. B cells from these mice will be assessed for alterations in signaling and function.

Chapter 5: CONCLUSION

Current results show that depletion of one of the specific products of PI3K, PI(3,4)P2, can selectively affect the activation of downstream phosphoinositide-binding proteins *in vitro*. It was shown that Akt's binding ability to the PM through its PH-domain is impeded thus inhibiting its full activation. Select downstream targets, such as GSK-3β and PRAS40, were also negatively affected from the decrease in Akt activation and function, however the other downstream targets mTORC1 and p70-S6K were not affected. The negative effects of Akt and select downstream target phosphorylation translated to a decrease in cellular processes, specifically glycolytic metabolism, indicating the overexpression of INPP4A in B cells does have some adverse effects on BCR activity and B cell metabolism. The second branch of the PI3K pathway, Btk and PLCγ, and the subsequent calcium signaling response during BCR activation was not affected when INPP4A was overexpressed in transduced Bjab B cell lines. Future experiments will further define the mechanisms by which PI(3,4)P2 depletion selectively affects these downstream signaling events and determine the impact on metabolic programming and B cells function *in vivo*. All in all, these results are the first to describe the effects how PI(3,4)P2 hydrolysis affects specific downstream signaling pathways and how dysregulated INPP4A plays a role in regulating lymphocyte activation and metabolism in a B lymphocyte cellular context and overall filling more of the knowledge gap in understanding BCR activation.

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