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The Impact of Phosphoinositide Metabolic Enzymes in Glioblastoma: a tale of Phosphoinositide-Specific Phospholipase C PLCβ1 and 5-Phosphatase SKIP

Presentata da: Dott. Eric Owusu Obeng

Coordinatore Dottorato: Chiar.mo Prof. Pietro Cortelli

Supervisore Chiar.mo Prof. Lucio Ildebrando Cocco

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Table of Contents

Abstract

Background: Glioblastoma multiforme (GBM) is one of the deadliest and most aggressive form of primary brain tumor. Unfortunately, current GBM treatment therapies are not effective in treating GBM patients. They usually experience very poor prognosis with a median survival of approximately 12 months. Only 3-5% survive up to 3 years or more. A large-scale gene profile study revealed that several genes involved in essential cellular processes are altered in GBM, thus, explaining why existing therapies are not effective. The survival of GBM patients depends on understanding the molecular and key signaling events associated with these altered physiological processes in GBM. Phosphoinositides (PI) form just a tiny fraction of the total lipid content in humans, however they are implicated in almost all essential biological processes, such as acting as second messengers in spatio-temporal regulation of cell signaling, cytoskeletal reorganization, cell adhesion, migration, apoptosis, vesicular trafficking, differentiation, cell cycle and post-translational modifications. Interestingly, these essential processes are altered in GBM. More importantly, incoming reports have associated PI metabolism, which is mediated by several PI phosphatases such as SKIP, lipases such as PLCβ1, and other kinases, to regulate GBM associated cellular processes. Even as PLCβ1 and SKIP are involved in regulating aberrant cellular processes in several other cancers, very few studies, of which majority are in-silico-based, have focused on the impact of PLCβ1 and SKIP in GBM. Hence, it is important to employ clinical, *in vitro*, and *in vivo* GBM models to define the actual impact of PLCβ1 and SKIP in GBM.

AIM: Since studies of PLCβ1 and SKIP in GBM are limited, this study aimed at determining the pathological impact of PI metabolic enzymes, PLCB1 and SKIP, in GBM patient samples, GBM cell line models, and xenograft models for SKIP.

Results: For the first time, this study confirmed through qPCR that PLCβ1 gene expression is lower in human GBM patient samples. Moreover, PLCβ1 gene expression inversely correlates with pathological grades of glioma; it decreases as glioma grades increases or worsens. Silencing PLCβ1 in U87MG GBM cells produces a dual impact in GBM by participating in both pro-tumoral and anti-tumoral roles. PLCβ1 knockdown cells were observed to have more migratory abilities, increased cell to extracellular matrix (ECM) adhesion, transition from epithelial phenotype to mesenchymal phenotype through the upregulation of EMT transcription factors Twist1 and Slug, and mesenchymal marker, vimentin. On the other hand, p-Akt and p-mTOR protein expression were downregulated in PLCβ1 knockdown cells. Thus, the oncogenic pathway PI3K/Akt/mTOR pathway is inhibited during PLCβ1 knockdown. Consistently, cell viability in PLCβ1 knockdown cells were significantly decreased compared to controls. As for SKIP, this study demonstrated that about 48% of SKIP colocalizes with nuclear PtdIns(4,5)P_2 to nuclear speckles and that SKIP knockdown alters nuclear PtdIns(4,5)P_2 in a cell-type dependent manner. In addition, SKIP silencing increased tumor volume and weight in xenografts than controls by reducing apoptosis and increasing viability. All in all, these data confirm that PLCβ1 and SKIP are involved in GBM pathology and a complete understanding of their roles in GBM may be beneficial.

Introduction

1. Phosphoinositides

Lipids, especially, phospholipids, are the primary structural component of all mammalian cellular membranes. Phospholipids include phosphoinositides (PIs), phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, sphingomyelin and phosphatidic acid (Cheng et al., 2016; Van Meer et al., 2008). PIs represent one of the most studied phospholipids. They comprise of the precursor phosphatidylinositol (PtdIns) and their phosphorylated forms, polyphosphoinositides (PPI). PtdIns is composed of a diacylglycerol (DAG) backbone which is phosphodiesterified to a 6-carbon cyclic polyol, that is the myo-inositol $(CHOH)_6$ head group. The DAG backbone is esterified at its *sn*-1 and *sn*-2 positions to two fatty acids and it is linked via a phosphate at its *sn*-3 position to the D1 position of the inositol head group.The myo-inositol head group of PtdIns bears 5 equatorial and one axial hydroxyl groups which confers a turtle structure or conformation (Figure 1) (Agranoff, 1978; Balla, 2013).

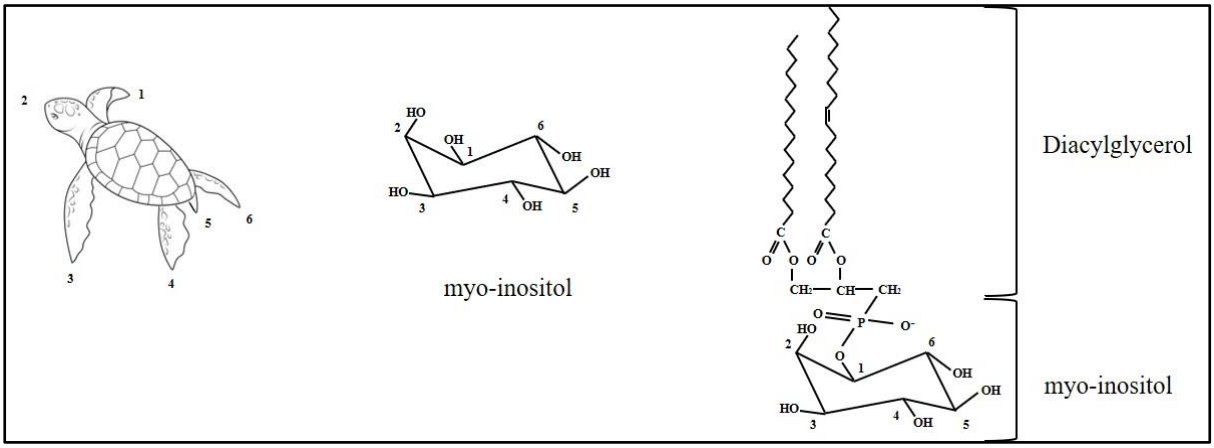


Figure 1: *The phosphatidylinositol structure according to Agranoff's concept: A scheme representing the positions of the specific hydroxyl groups in the myo-inositol ring following the turtle concept by Agranoff. The DAG backbone is phosphodiesterified to a 6-carbon cyclic polyol, the myo-inositol $(CHOH)_6$ head group (Owusu Obeng et al., 2020).*

Phosphorylation can occur on three out of five equatorial hydroxyl groups at positions -3,-4,-5 on the inositol ring of PtdIns to generate the seven established PPIs, namely: PtdIns(3,4,5)P_3, PtdIns(4,5)P_2, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns3P, PtdIns4P and PtdIns5P (Figure 2) (Owusu Obeng et al., 2020). The fraction of PIs is relatively tiny compared to all other phospholipids in eukaryotic cell membranes. However, they are implicated in almost all essential biological processes acting as second messengers in spatio-temporal regulation of cell signaling, cytoskeletal reorganization, cell adhesion, migration, apoptosis, vesicular trafficking, differentiation, cell cycle and post-translational modifications (Balla, 2013; Bandu et al., 2018; Godi et al., 2004; Shevchenko and Simons, 2010).

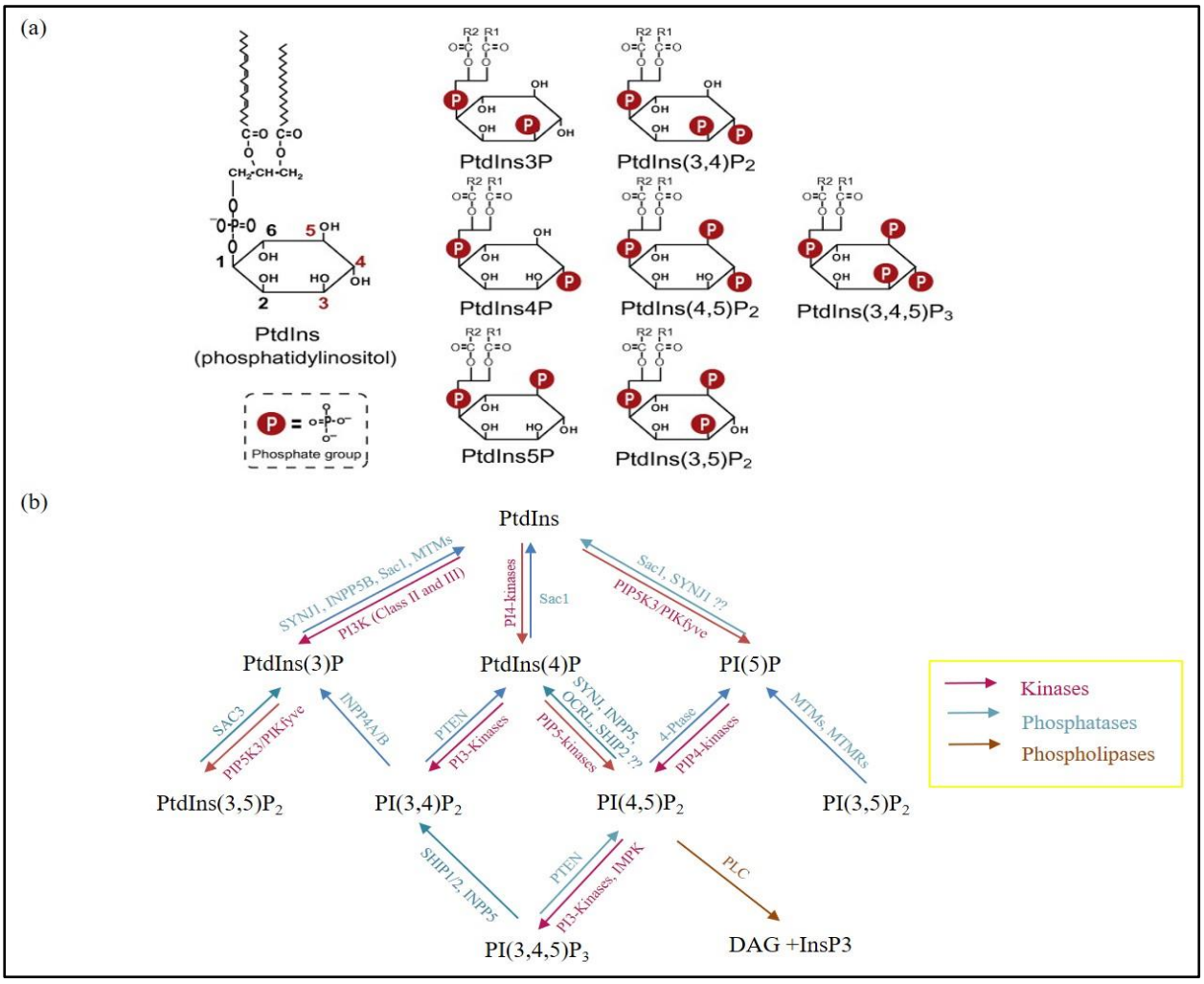


Figure 2: *Polyphosphoinositides and phosphatidylinositol metabolism. (a) PtdIns is the primary substrate of all the seven recognized PPIs. PtdIns generates PPIs with distinct functions and distribution through the activities of several PI metabolic enzymes at various equatorial hydroxyl positions (Sasaki et al., 2009). (b) A representation of how PtdIns is regulated by PI specific metabolic enzymes to yield the seven different PPIs. These enzymes include several lipases, phosphatases and kinases which catalyze PI-dependent reactions. ?? corresponds to PI-dependent metabolic enzymatic reactions that are not completely understood (Owusu Obeng et al., 2020).*

Distribution of PIs in mammalian cells varies and may not be limited to only one organelle, hence, they localize to specific sites. For instance, PtdIns(4)P in the Golgi, PtdIns(3)P in the endosomes, PtdIns(4,5)P_2 in the cytoplasm (Balla, 2013; Di Paolo and De Camilli, 2006; Shewan et al., 2011; Wymann and Schneiter, 2008). In addition to the typical cytoplasmic localization, PIs are also found in the nucleus, where they are regulated independently (Ratti et al., 2017). PIs are regulated by several metabolic enzymes including phospholipases, phosphatases and kinases which contributes to so-called 'PI cycle'. Interestingly, nuclear PIs localize in the nuclear sub-compartments together with these metabolic enzymes. This explains why nuclear PI signaling is separate from the cytoplasmic PI signaling (Ratti et al., 2017; Xian et al., 2020).

1.1 Nuclear Phosphoinositides

The first evidence of PIs was demonstrated in the 1950s (HOKIN and HOKIN, 1953). All attention or studies on PIs were geared towards understanding the functional mechanisms of cytosolic membrane-associated PIs until, after about a decade, the presence of a phospholipid content in the nuclear chromatin was reported (Manzoli et al., 1977; Rose and Frenster, 1965). This was a rather interesting era for phospholipid research, inspiring a mass interest in nuclear phospholipid research. In 1983, also phosphatidylinositol phosphate kinase activity was detected in the nucleus (Smith and Wells, 1984), paving way for a subsequent discovery of a distinct PI cycle in the nucleus (Cocco et al., 1987; Divecha et al., 1991; Payrastre et al., 1992).

An increasing number of reports demonstrate that the nucleus is a highly organized organelle which is comprised of several membraneless and dynamic sub-nuclear compartments (Nunes and Moretti, 2017; Spector and Lamond, 2011; Wilson and Dawson, 2011). In addition, chromosomes are not distributed randomly in the nucleus: they are distributed across distinct regions, called chromosome territories. The chromosomes then interact with various sub-nuclear compartments such as nucleoli, promyelocytic leukemia bodies, nuclear speckles, paraspeckles, nuclear pore complexes, Cajal bodies and nuclear lamina (Figure 3) (Nunes and Moretti, 2017; Zhao et al., 2009).

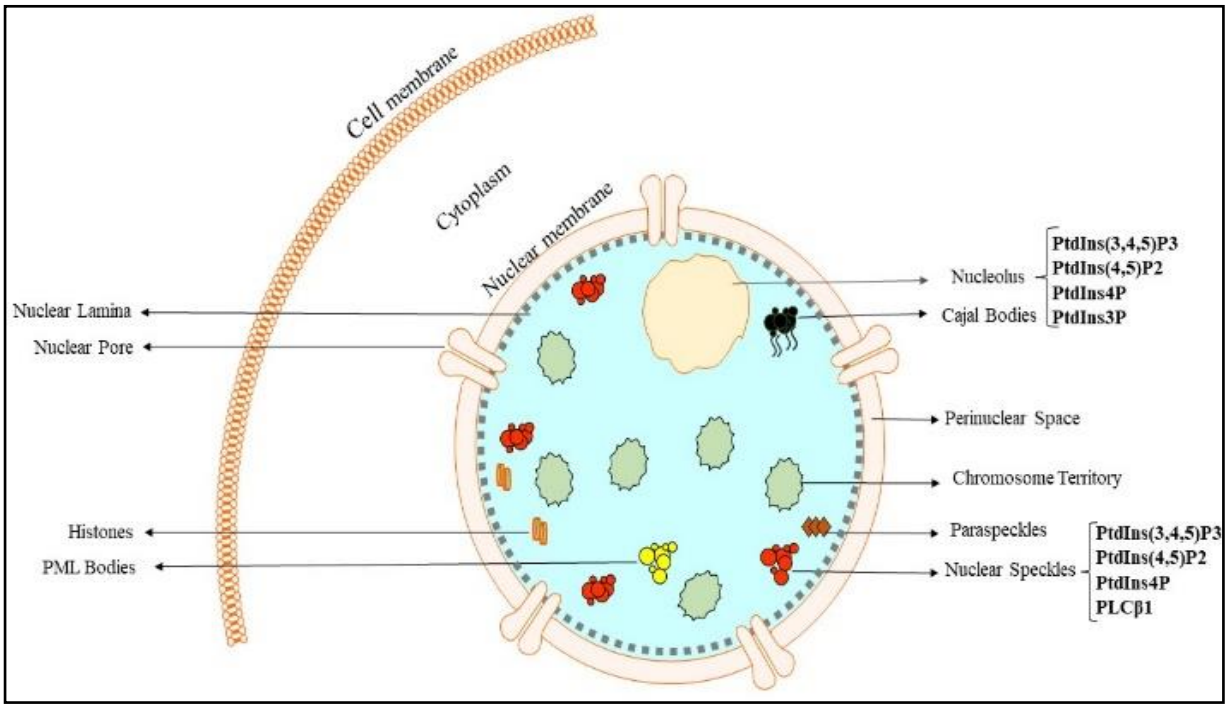


Figure 3: *The nuclear structure showing nuclear sub-compartment and localization of PIs. The nucleus is an extremely organized organelle organized into several membraneless sub-compartments. PIs localize to these sub-compartments together with their metabolic enzymes to regulate cell signaling independent of cytosolic PI signaling (Xian et al., 2020).*

The notion that localization of key molecules to the nucleus is non-random may explain why several incoming reports suggest that gene expression may be influenced by nuclear positioning (Fiume et al., 2015; Jacobsen et al., 2019; Zhao et al., 2009). Thus, gene expression is affected by nuclear distribution and, as such, the nucleus must be highly organized.

Nuclear speckles are membraneless sub-nuclear compartments frequently presented as 20-50 punctate irregular structures of varying shapes and sizes under fluorescent microscopes. They are situated in the interchromatin regions of the nucleus that houses transcription regulatory proteins and pre-messenger RNA splicing factors that are often recruited to active transcription sites (Spector and Lamond, 2011; Zhao et al., 2009). This may confirm why genes that localize within nuclear speckles are frequently involved in gene expression. Indeed, nuclear PIs function as cofactors of several nuclear processes, including DNA repair and regulation of transcription, with a pathological relevance. Notably, an imbalance of the signaling pathways associated with a change in the composition of nuclear PIs can result in chronic inflammatory diseases, cancer and degenerative syndromes (Cocco et al., 2015a; Follo et al., 2013; Ratti et al., 2019a).

Detection of PIs, especially nuclear PIs, remains one of the greatest challenges in this field. Current methods of detecting PI *in vivo* employ the use of light and electron microscope to visualize antibodies and PI-binding domains that target specific PIs (Kalasova et al., 2016). Thanks to the use of various modern and advanced techniques, all PIs, except PtdIns(3,5)P2, have been shown to localize in the nucleus (Table 1) (Fiume et al., 2015; Jacobsen et al., 2019), across multiple nuclear subcompartments, such as speckles and nucleoli (Chen et al., 2020).

Table 1. Sub-compartmental localization of Nuclear PIs (Xian et al., 2020).

Phosphoinositides	**Nuclear Localization**
PtdIns(3,4,5)P3	Matrix, nucleoplasm, speckles, Nucleoli
PtdIns(4,5)P2	Speckles, nucleoli, nucleoplasm, nuclear lipid islets
PtdIns(3,4)P2	Speckles, nuclear membrane
PtdIns5P	Chromatin and matrix
PtdIns4P	Nucleoli, speckles, chromatin
PtdIns3P	Nucleoli, matrix

1.2 Nuclear processes controlled by nuclear PIs

Using qualitative mass spectrometry, it was shown that nuclear PIs bind to over 120 nuclear proteins which exhibit varied affinities for nuclear PIs (Jungmichel et al., 2014). Nuclear PIs, in turn, regulate several nuclear processes through the bond with numerous nuclear proteins.

1.2.1. Gene Expression

Gene expression entails a series of tightly controlled nuclear events, including RNA processing and RNA shuttling into the cytoplasm. Further processing on precursor RNA is conducted before mature RNA is transported into the cytoplasm and these include 5' capping, 3' cleavage, polyadenylation, and RNA splicing (Osborne et al., 2001). Accumulating evidence link the control of these nuclear processes to nuclear PIs. For instance, an *in vitro* study demonstrated the involvement of nuclear PtdIns(4,5)P_2 in pre-mRNA splicing and gene transcription. It appeared that there was an interaction among nuclear PtdIns(4,5)P_2, the nuclear speckle protein SC-35 and the hyperphosphorylated form of the large unit of RNA polymerase II (RNA pol II). As a matter of fact, via immunodepletion, PIs limited pre-mRNA splicing (Osborne et al., 2001). Furthermore, Mellman *et al.* employed a yeast-two hybrid screen model to detect nuclear PIPKIα interacting proteins. This study demonstrated the interaction between the poly (A) polymerase called Star-PAP (speckle targeted PIPKIα regulated-poly (A) polymerase) and nuclear PtdIns(4,5)P2 (Mellman et al., 2008). Importantly, this interaction induces a nuclear PtdIns(4,5)P_2 mediated polyadenylation, that is, promoting the initiation and elongation steps. In addition, nuclear IPMK (Human inositol polyphosphate multikinase) has also been shown to regulate a transcript-selective nuclear mRNA shuttling to the cytoplasm via PtdIns(3,4,5)P_3 generation (Wickramasinghe et al., 2013).

1.2.2 Chromatin Remodelling

The chromatin complex includes histone proteins tightly wounded to DNA, and eukaryotic DNA is highly condensed in the nucleus to form this complex. Gene expression is a highly controlled process regulated by chromatin remodelling. Several studies have showcased the impact of some nuclear PIs on chromatin opening. For example, nuclear PtdIns(4,5)P_2 regulates chromatin remodelling through its association with the SW1/SNF-like/ BAF (BRM-associated factors) chromatin remodelling complex, this is made possible via its ATPase sub-component BRG1 (Zhao et al., 1998). Notably, nuclear PtdIns(4,5)P_2interacts directly with H1

and H3 histone proteins. Numerous incoming studies have reported the capability of H1 histone proteins to restrict the activity of RNA polymerase II during transcription and, intriguingly, this is not completely reversed upon adding back PtdIns(4,5)P_2 (Croston et al., 1991; Yu et al., 1998). On the other hand, nuclear PtdIns5P also controls chromatin remodelling through its association with the histone code reader related to the chromatin complex ING2 (Inhibitor of growth protein 2). Of note, ING2 is a subunit of the HDAC1 complex (Sin3a- histone deacetylase 1) which is implicated in epigenetic gene expression. Thus, PtdIns5P regulates ING2 binding with the chromatin complex as well as potentially regulating epigenetic gene expression and DNA damage (Bua et al., 2013; Castano et al., 2019).

1.2.3. Cell Survival

PtdIns(3,4,5)P_3 is a major signaling axes reported to be involved in cell survival through promotion of the expression of anti-apoptotic signals (Castano et al., 2019). Nerve growth factor (NGF) stimulates nuclear PtdIns(3,4,5)P_3 in order to regulate cell survival by interacting with nucleophosmin (B23) to form the PtdIns(3,4,5)P_3-B23 complex. This complex restricts DNA fragmentation by inhibiting the nuclease activity of caspase-activated DNase (CAD) and also protects cells from proteolytic cleavage by communicating with nuclear protein kinase B (PKB/Akt) (Ahn et al., 2005; Sang et al., 2008). All in all, these evidences highlight the importance of understanding the signaling events associated with nuclear PIs and their effector proteins. Extending this knowledge to cancer and other pathologies may be beneficial for therapeutic interventions.

2. Phosphoinositide synthesis

The PI composition of eukaryotic cells are regulated by several lipid kinases, phosphatases and phospholipases that are activated upon response to different external stimuli. They regulate the PI cycle and a plethora of studies have reported that a dysfunction in the PI cycle as a result of deregulated mechanisms of these enzymes lead to several diseases including cancer, Alzheimer's disease, myelodysplastic syndromes (MDS), and infertility [12–14]. Due to the significant role of PIs in almost all mammalian cellular processes, the activities of PI metabolic enzymes, PI-specific kinases and phosphatases are tightly regulated through their association with regulatory proteins (Burke, 2018).

2.1 Phospholipases

Phospholipases are a group of PI metabolic enzymes that synthesize the molecular breakdown of phospholipids into biologically active lipid products that regulate several physiological or cellular functions (Follo et al., 2019c; Park et al., 2012; Ratti et al., 2020). So far, there have been the identification of only four major families of phospholipases, namely: phospholipases A, B, C, and D (PLA, PLB, PLC, and PLD, respectively). They have specific functions and are grouped into each family depending on the type of reaction they catalyze (Cho and Han, 2017). For instance, PLA isoforms generate free fatty acids and lysophospholipids via targeting of the glycerol constituent of phospholipids, while PLD isoforms catalyze the hydrolysis of phosphatidylcholine into choline and phosphatidic acid. Numerous studies on phospholipases are centred on specific sub-families. Notably, individual family members have been demonstrated to have specific targets and functions even though they may share common signaling pathways with other sub-family members (Cho and Han, 2017; Park et al., 2012). PLCs represent one of the most frequently studied phospholipases in cancer. However, their ability to regulate major cellular roles in the oncogenic process and the potential crosstalk among distinct PLCs continue to remain a mystery that needs unravelling.

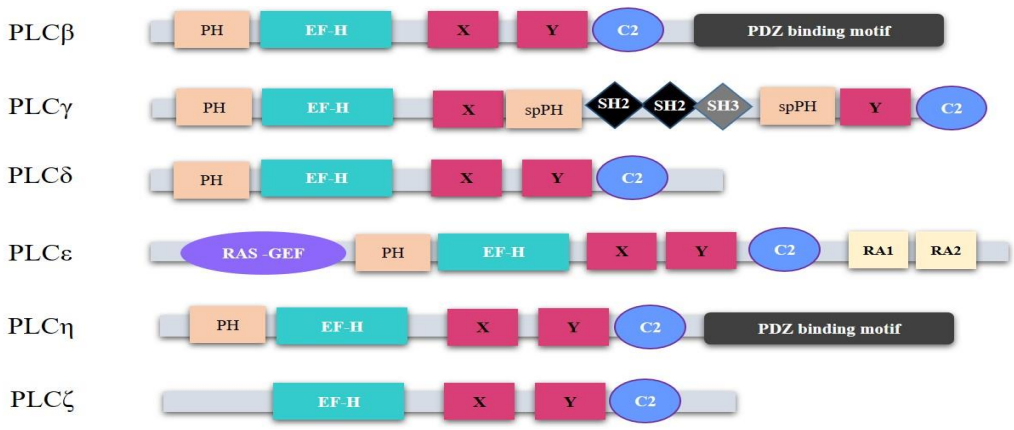
PLCs are made up of 6 sub-family members that catalyze the hydrolysis of PtdIns(4,5)P_2 into the two important intracellular second messengers diacylglycerol (DAG) and inositol 1,4,5 trisphosphate ($InsP_3$). A series of downstream signaling cascades are activated upon the generation of DAG and $InsP_3$. For example, this promotes the activation of protein kinase C (PKC) and the release of calcium ions (Ca^{2+}) from the intracellular stores, respectively (Faenza et al., 2003; Poli et al., 2016; Ratti et al., 2017b; Rhee, 2001). $InsP_3$ detaches from the membrane and interact with its receptors to regulate intracellular Ca^{2+} release from the endoplasmic reticulum, while DAG continues to bind to the membrane to induce the PKC activation upon Ca^{2+} release from the intracellular stores (Lyon and Tesmer, 2013). Of note, PLC-mediated signaling has been demonstrated to be central in the regulation of essential cellular processes associated with various cancer types. For instance, PKC is implicated in cell proliferation, differentiation, migration, and growth (Martelli et al., 2006). On the other hand, Ca^{2+} signaling has been shown to be critical in the regulation of cancer cell motility, division, and death (Hatziapostolou et al., 2011; Monteith et al., 2017). Simultaneously, the downregulation of PtdIns(4,5)P_2 expression via PLC-mediated hydrolysis also yields several

important signaling events related to cancer, especially cell migration. PtdIns(4,5)P_2 activity is a recognized regulator of several pleckstrin homology (PH) domain-containing proteins, as well as actin regulatory proteins, thus explaining its relevance in cancer cell migration (Rhee, 2001; Song et al., 2001).

2.2.1 Structure and Activation of PLCs Implicated in Cancer

PLCs are made up of a family of 13 distinct isozymes that grouped into six specific sub-families, namely: PLCβ (1,2,3 and 4), PLCγ (1 and 2), PLCδ (1,3 and 4), PLCε, PLCη (1 and 2), and PLCζ depending on their structural conservation and domain organization (Balla, 2013; Cocco et al., 2015a; Lyon and Tesmer, 2013; Owusu Obeng et al., 2020). Until now, all detected PLC isoforms possess conserved domains or regions such as the X and Y regions, making up the catalytic domain, the PH domain, the EF-hand (EF-H) motif and the PKC homology (C2) domain. All these domains play some functional roles in mammalian cells. The PH domain of PLCs promote their binding to PtdIns(4,5)P_2 with high affinity and specificity, the EF-H motif are associated with scaffolding roles that support guanosine triphosphate (GTP) hydrolysis upon G-protein coupled receptor (GPCR) binding while the C2 domain contributes to both intra- and inter-molecular signaling processes (Figure 5a) (Lyon and Tesmer, 2013; Rhee, 2001).

(a)



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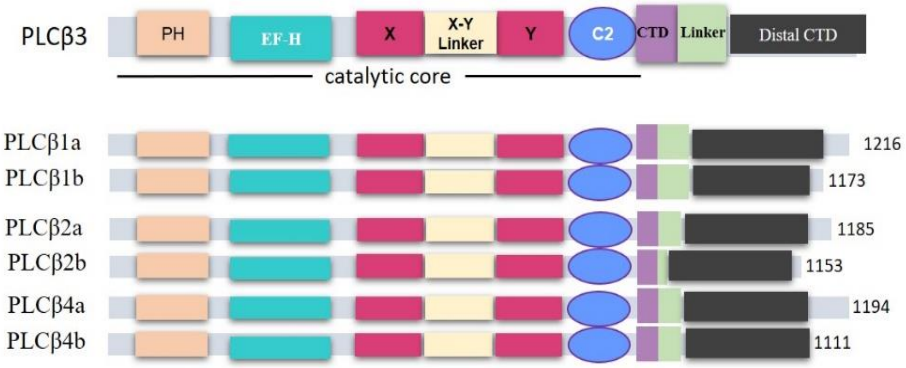

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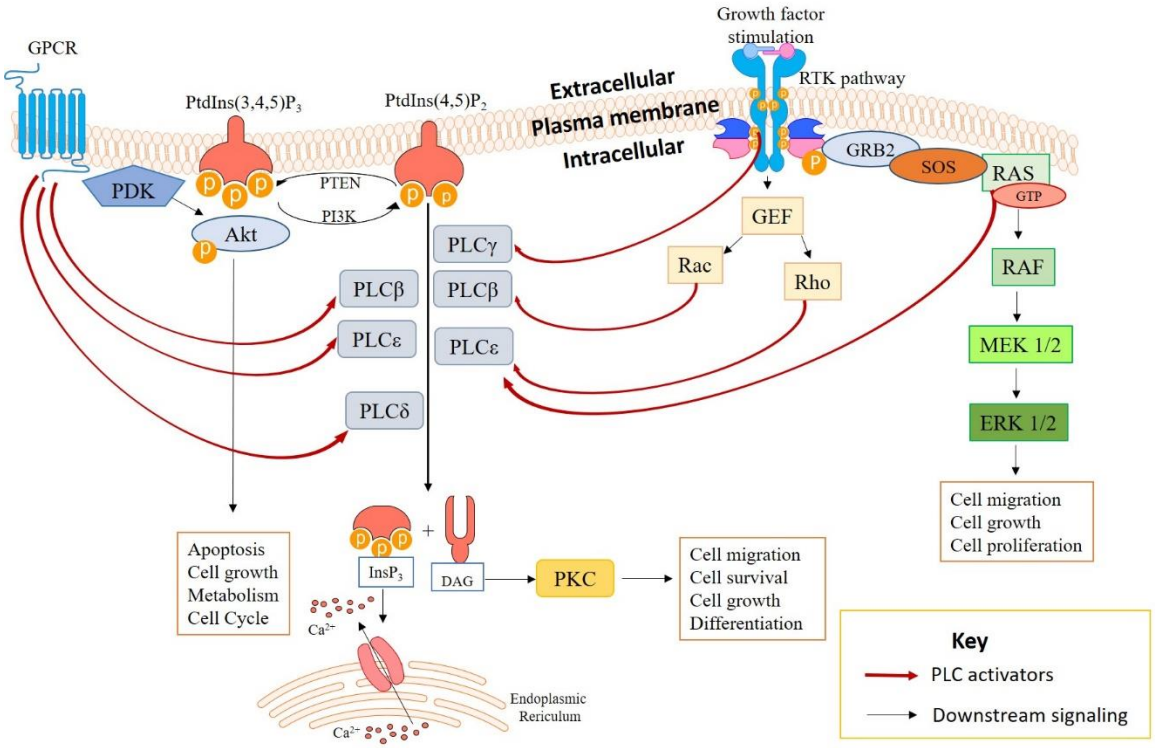


Figure 5. *The structural organization and activation of PLC family members. (a): Cartoon demonstrating the structural organization of the PLC isoforms. PLC family members exhibit structurally conserved regions like the EF-H, X / Y catalytic core, and the C2 domain. However, their regulatory domains are structurally organized following a PLC subtype dependent manner. (b): cartoon representation of the various PLCβ isoforms. PLCβ spliced variants possess structurally conserved features, they are distinguished by the differences present at*

their C-terminal regions (CTD) with varied lengths and sequences. (c): a scheme representing the activation of PLCs that are implicated in cancer. PLCs are activated by stimulations from several different cues to promote the catalyzation of PtdIns(4,5)P_2 hydrolysis into InsP_3 and DAG, which subsequently induces the intracellular release of Ca^{2+} from the ER as well as PKC activation, respectively. Activation of PKC and Ca^{2+}a release results in series of signaling events that regulate cell migration, cell survival and differentiation in cancer. Activation of some PLCs are through by multiple mechanisms. For instance, PLCε can be activated by multiple stimulating cues like the RAS, the RAS homolog family member (Rho), and G-proteins. PLCβ2 can also be activated by G-proteins pathway as well as the RAS-related C3 botulinum toxin substrate (Rac) GTPases. These activation mechanisms may be cell type specific or stimuli dependent. Red coloured signs show reactions that activate PLCs while black signs show downstream signaling paths (Owusu Obeng et al., 2020).

As previously suggested, PLC family members may show conservations in structural organizations. However, regulatory domains like PH domain, rat sarcoma (Ras) associating domain (RA domain), C2 domain and the EF-H domain specifically organized in a PLC subtype manner, thus, explaining why individual PLC family members possess unique functions and cellular distribution (Cocco et al., 2015b; Owusu Obeng et al., 2020). Numerous studies have demonstrated that PLCs are uniquely distributed across several cellular sub-compartments together with their respective substrate PIs. For example, due to localization of PIs to the nucleus, some PLC isoforms like Some PLC isoforms like PLCβ1 (Cocco et al., 1999; Follo et al., 2014), PLCγ1 (Liu et al., 2014), PLCδ1 (Jonathan D. Stallings et al., 2005), PLCδ4 (Kunrath-Lima et al., 2018), and PLCε (Zhang et al., 2013) have also been shown to localize to the nucleus to regulate the nuclear PI metabolism or cycle which is independent of the normal cytosolic PI cycle (Manzoli et al., 1995; Owusu Obeng et al., 2020; Ratti et al., 2017b; Xian et al., 2020). Essential to this study, there are several demonstrations of the involvement of nuclear PLCs in cancer (Manzoli et al., 2014; Mongiorgi et al., 2016; Ratti et al., 2019b).

2.2.2 PLCβ

The PLCβ subfamily of PLCs represents one of the most frequently studied forms of PLCs and it is made up of 4 isoforms, namely: PLCβ1, PLCβ2, PLCβ3, and PLCβ4 (Cocco et al., 2015b).

Some of these isoforms also exist as spliced variants. For example, PLCβ1: a and b, PLCβ2: a and b, and PLCβ4: a and b. PLCβ isoforms possess structurally conserved domains with minor variations, especially at the C-terminal domain. The length and sequences of the C-terminal region of the various isoforms vary, some are shorter than others (Figure 5b) (Lyon and Tesmer, 2013). Isoforms of PLCβ are activated via the classical G-protein route, by the Gαq- and Gβγ subunits of heterotrimeric G proteins (Cocco et al., 2015b). However, for PLCβ2, Rac, which is a GTPase from the Rho family, has been shown to also activate it, in part due to Rac high affinity for binding to the PH domain of PLCβ2 (Illenberger et al., 2003) (Figure 5c). Notably, PLCβ1 is regarded as the most studied PLCβ isoform which is expressed in the nervous system, specifically in the cerebral cortex and hippocampus (Böhm et al., 2002), and in the cardiovascular system (Arthur et al., 2001; Mende et al., 1999). Instead, PLCβ2 isoforms are expressed in hematopoietic cells and platelets, where they control chemotaxis s (Lyon and Tesmer, 2013; Suh et al., 2008). PLCβ3 also localizes within the liver, brain, hematopoietic cells and the cardiovascular system and regulates cell proliferation and chemotaxis (Lyon and Tesmer, 2013). Lastly, PLCβ4 localizes within the cerebellum as well as in the retina to promote visual processing after phototransduction (Jiang et al., 1996).

2.3 PLCs in cancer development and progression

Since PLCs are significantly involved in mediating several critical cellular processes, deregulations in their activities and expressions may be fatal to mammalian cells. In fact, alterations in PLC activities have been demonstrated in different human cancer types through *in vitro, in vivo* and clinical studies. For instance, PLCβ involvement in hematopoietic malignancies and neuroendocrine tumors (Folio et al., 2009; Follo et al., 2019a; Lu et al., 2016; Sengelaub et al., 2016),

2.3.1. PLCs in cancer cell proliferation, survival, and tumor growth

PLC activities interact with major pathways often deregulated in cancer such as the PI3K/Akt/mTOR (Wang et al., 2018), RAS/RAF/MAPK/ERK (Zhang et al., 2020), and JAK/STAT (Xiao et al., 2009) pathways which are critical signaling hubs for the regulation of cancer cell growth and proliferation (Figure 6).

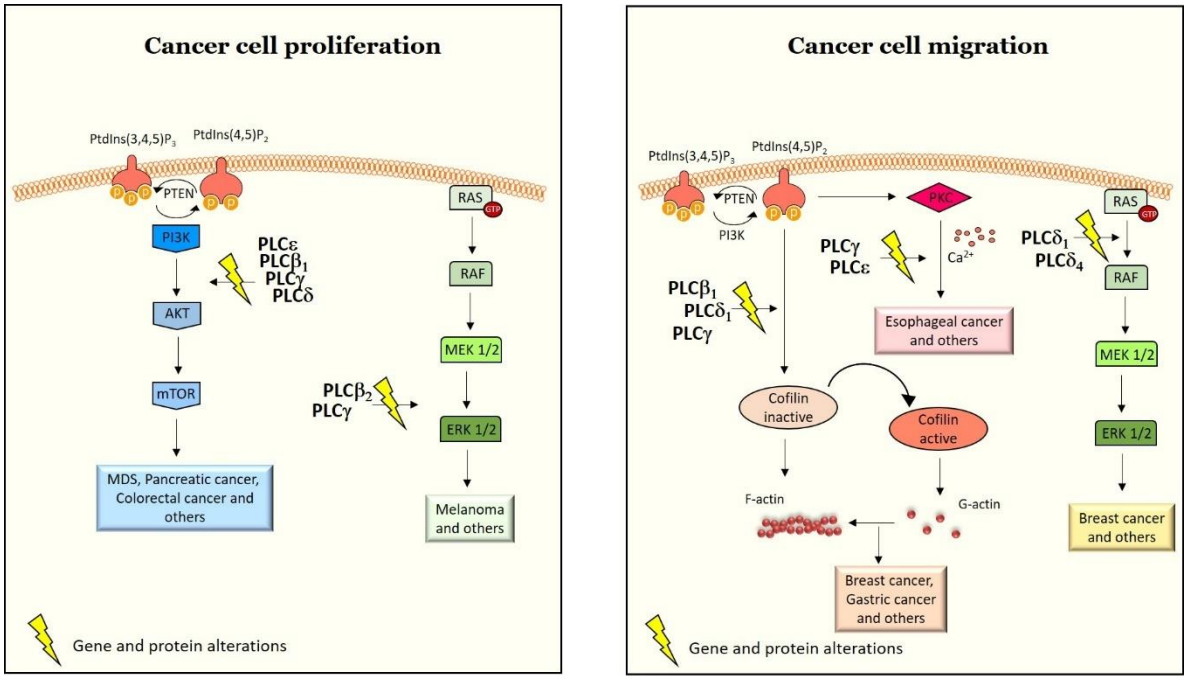


Figure 6. *Schematic representation of PLC-dependent cell proliferation and migration in cancer. Currently, all PLCs except PLCζ and PLCη, have been reported to regulate cancer cell proliferation and migration. Alterations associated with the expression of PLC isoforms in diverse cancer subtypes have been demonstrated to influence the PI3K/Akt/mTOR and the RAS/RAF/MAPK/ERK pathways which dominates in the regulation of cell survival, growth and proliferation. Similarly, alterations in PLC isoforms also regulates actin reorganization through cofilin activation which is essential for cell migration. Furthermore, alterations in PLCδ expression affects cell migration in breast cancer cells via ERK signaling. Yellow lightning symbol represents molecular alterations in gene or protein expression (either upregulation or downregulation) (Owusu Obeng et al., 2020).*

2.3.2. PLCs in Cell migration, invasiveness, and metastasis

Numerous deaths associated with cancer are primarily related to the migratory or metastatic potential of tumors to invade new regions different from original tumor sites. Single or tumor cell clusters separate from primary tumors, assume migratory abilities, and invade new regions via their surrounding matrices. Interestingly, PLC isoforms have been reported to participate in this process in several cancer types (Bertagnolo et al., 2007; Chen et al., 1996; Sengelaub et al., 2016).

First and foremost, it been demonstrated that PLCβ1 communicates with the Protein Tyrosine Phosphatase Receptor Type N2 (PTPRN2) protein to mediate metastasis in breast cancer cell lines. There is an increased expression of both proteins which correlates positively with metastatic relapse in breast cancer patients. The authors revealed that PLCβ1 and PTPRN2 regulates cell migration in breast cancer cells by stimulating a decrease in plasma membrane PtdIns(4,5)P_2 levels (Sengelaub et al., 2016). Notably, PtdIns(4,5)P_2 is an established PLCβ1 substrate which is known to control actin dynamics and cell migration (Audhya and Emr, 2003; Fiume et al., 2019). Sengelaub and colleagues reported that the decrease in plasma membrane PtdIns(4,5)P_2 levels in breast cancer cells activates the actin binding factor, cofilin, by stimulating its release into the cytoplasm where it regulates actin turnover dynamics to enhance cell migration and metastasis (Sengelaub et al., 2016; Stevenson et al., 2012).

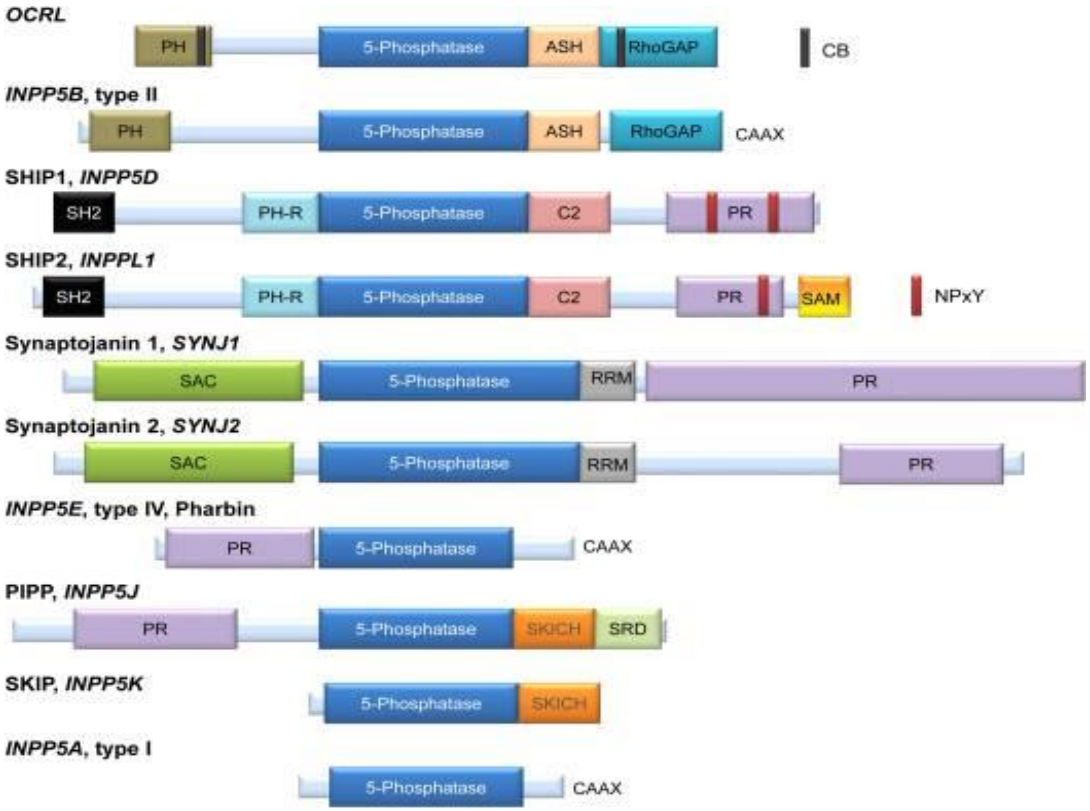
2.4 PI 5-Phosphatases (INPP5Ps) in cancer

Human PI 5-phosphatases are made up of a family of 10 members that share conserved features such an inositol 5-phosphatase domain and several amino acids which makes them possess comparable structures (Figure 7a) (Ramos et al., 2019b). All the members possess a phosphoinositide phosphatase activity except INPP5A which acts solely on soluble inositol polyphosphates. Although the other members can also dephosphorylate soluble inositol polyphosphates, their main function is to catalyze the dephosphorylation at the 5 position of lipid-bound inositol polyphosphoinositides (Dong et al., 2018; Ramos et al., 2019b). For instance, they catalyze the dephosphorylation of PtdIns(3,4,5)P_3, PtdIns(3,5) P_2, and PtdIns(4,5)P_2 at the 5-phosphate position of the inositol ring into PtdIns(3,4)P_2, PtdIns3P, and PtdIns4P respectively (Figure 7b). All the nine members with phosphatase activities are established cytosolic enzymes with catalytic modules that are flanked by functional domains which controls their subcellular targeting to membranes where they express their catalytic action (Dong et al., 2018).

They include: Src homology domain 2-containing inositol 5-phosphatases (SHIP) 1 and 2, skeletal muscle and kidney enriched inositol phosphatase (SKIP)/INPP5K, oculocerebrorenal syndrome of Lowe (OCRL), INPP5A, INPP5B, synaptojanins (SYNJs) 1 and 2, INPP5E, and INPP5J (Ramos et al., 2019b). Hence, they are recognized negative

controllers of PtdIns(3,4,5)P_3 which may produce tumor suppressor effects in cancer cells (Toker and Rameh, 2015), as well as a positive regulators of PI(3,4)P_2 (Ramos et al., 2019b). In this study, we focus on establishing the effects and localization of SHIP2 and INPP5K/SKIP in glioblastoma cells and in vivo models.

(a)



(b)

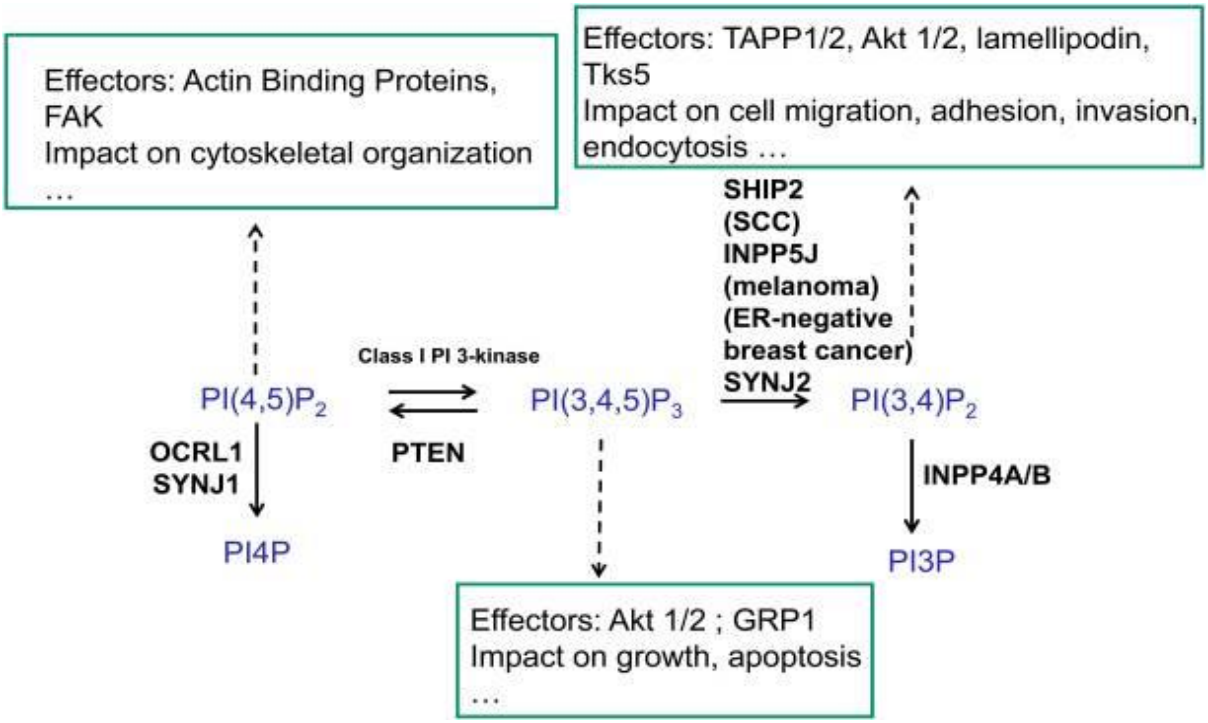


Figure 7: *Structural representation and signaling of PI 5-phosphatases. (a) INPP5Ps share several conserved domains and structure, however, some are very similar compared to others. For instance, OCRL and INPP5B possess the same internal structural organization with few differences at the C terminus; SHIP1 and SHIP2 also differs just at the C terminus. INPP5A represents the smallest member of the INPP5P family and it is made up of the 5-phosphatase domain and a CAAX sequence at the C terminus (b) schematic representation of the signaling effects produced by human INPP5Ps. Cellular processes such as survival, focal adhesion, cell migration and invasion are mediated by INPP5P synthesis of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2* (Ramos et al., 2019b).

It has been demonstrated that different PI-5 phosphatases mediate either tumorigenic or tumor suppressor functions, or even both. The reason for this discrepancy demonstrated in several cancer models is unknown. This may be associated with the activity/specificity or non-catalytic functions of the different PI 5-phosphatases that also interact with diverse proteins (Ramos et al., 2018).

2.4.1 SHIP2

SHIP2 is encoded by the *INPPL1* gene located on human chromosome 11 and it is ubiquitously expressed in several different human tissues, showing an increased mRNA expression in human skeletal muscle, placenta, and the heart (Pesesse et al., 1997). A study in mouse revealed demonstrated that SHIP2 mRNA was expressing in all analyzed tissues (Schurmans et al., 1999). SHIP1, which is structurally close to SHIP2 with about 38% sequence identity is mainly restricted to hematopoietic lineages and during spermatogenesis (Liu et al., 1998). SHIP2 possesses several conserved motifs, including the amino terminus Src homology 2 (SH2) domain, the 5-phosphatase domain, and the carboxyl terminus proline rich and sterile α motif (SAM) domains (Figure 7a) (Lehtonen, 2020; Ramos et al., 2019b). The preferred substrate of SHIP2 is PtdIns(3,4,5)P_3, but it has also been found to control plasma membrane PtdIns(4,5)P_2, thereby participating in the control of cell migration in 1321 N1 glioblastoma cells (Ramos et al., 2018). Localization of SHIP2 to cells is complex because it is dynamic and varies with response to various agonists. It has been shown to localize to nucleus, plasma membrane, and ruffles (Ramos et al., 2019b).

Furthermore, SHIP2 has numerous tyrosine, serine, and threonine phosphorylation regions (Lehtonen, 2020; Thomas et al., 2017). The catalytic phosphatase activity of SHIP2 remains controversial due to the several different reports claiming how it is regulated. It has been shown that SHIP2 catalytic activity is regulated via its tyrosine phosphorylation, as well as via phosphorylation of its serine or threonine sites, thus, making this complicated to understand the true path of its activation (Lehtonen, 2020; Thomas et al., 2017). Just as other members of the PI 5-phosphatase family, SHIP2 phosphatase activity negatively regulates AKT activation as well as other independent functions which it regulates upon interacting with numerous recognized partners. For example: it regulates cell spreading, adhesion, migration, actin cytoskeleton dynamics, vesicular trafficking, endocytosis, FGF and EGF-mediated signaling, apoptosis and PI3K-mediated insulin signaling (Blero et al., 2001; Erneux et al., 2011; Lehtonen, 2020).

To understand the impact of SHIP2 in diseases, SHIP2 mutations have been reported to cause opsismodysplasia, which is a rare disease characterized by abnormal bone maturation (Huber et al., 2013). In addition, SHIP2 activity has been demonstrated to be

involved in diabetes, atherosclerosis, neurodegenerative diseases, and several cancer types (reviewed in (Lehtonen, 2020; Osborn et al., 2017; Ramos et al., 2019b)).

The fact that both SHIP2 and PTEN act on the same substrate reinforces its potential in cancer. Elevated SHIP2 expression is frequently reported in breast cancer, HCC, non-small cell lung cancer, and colorectal cancer, where SHIP2 expression correlates with poor survival of patients (Fu et al., 2013; Yang et al., 2014; Ye et al., 2016). SHIP2 has been demonstrated to promote breast cancer cell proliferation and tumor metastasis by interacting with c-cbl to prevent EGFR dynamics, thereby enhancing EGF-induced Akt activation (Prasad et al., 2008). In addition, SHIP2 has also been shown to activate Akt, JNK and promote the upregulation of the EMT marker vimentin in ER-negative breast cancer stem cells (Fu et al., 2014). Conversely, miRNA-205 targets SHIP2 in squamous cell carcinoma epithelial cells to promote Akt activation and inhibit apoptosis (Yu et al., 2008). It has also been demonstrated that overexpressing SHIP2 in glioblastoma cells inhibits Akt activation and results in cell cycle arrest (Taylor et al., 2000).

SHIP2 expression is frequently downregulated in gastric cancer relative to normal adjacent gastric mucosa (Ye et al., 2016). Intriguingly, overexpressing SHIP2 in gastric cancer cells suppresses cell proliferation, induced apoptosis, inhibited cell migration and invasion. In *in vivo* gastric tumor models, it inhibited xenograft growth (Ye et al., 2016). Moreover, silencing of SHIP2 expression in normal gastric epithelial cells supported anchorage-independent growth. However, overexpressing SHIP2 induced inactivation of Akt, and increased expression levels of the pro-apoptotic protein Bim, p21, and p27. Essentially, the restoration of Akt phosphorylation or activation in the gastric cancer cells markedly reduced the SHIP2-mediated inhibition of PI3K/Akt signaling, thereby reversing the inhibitory effect of SHIP2 on tumorigenesis and proliferation (Ye et al., 2016).

A report demonstrated that SHIP2 protein expression is upregulated in several breast cancer cell lines, which contrasts with PTEN expression (Prasad et al., 2008). Using SHIP2 siRNA to knockdown SHIP2 expression in MDA-231 cells showed a decrease in EGFR levels by means of enhanced receptor degradation. Furthermore, endogenous SHIP2 expression in MDA-MB 231 breast cancer cells promotes *in vitro* cell proliferation as well as increasing cellular sensitivity to drugs that target EGFR, promoting cancer

development and metastasis in nude mice. Moreover, it was also shown that a significantly high proportion of breast cancer clinical tissues (44%; *P* = 0.0001) contain elevated expression of SHIP2 protein specimens in contrast with non-cancerous breast tissues (Prasad et al., 2008).

2.4.2 SKIP/INPP5K

Human SKIP is encoded by the INPP5K gene, which is located on chromosome 17p 13.3, which is distal to *TP53* in human tumors. This chromosomal region is frequently reported to be hypermethylated or deleted in several human cancer types including breast, brain and HCC (Beckmann et al., 1997; Hedberg Oldfors et al., 2015). Several studies have demonstrated that SKIP localizes to the nucleus, plasma membrane, ruffles and the endoplasmic reticulum (ER) (Ijuin et al., 2000; Ramos et al., 2020). Recombinant full-length INPP5K possesses a 5-phosphatase activity toward both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, however, its preferred substrate is PtdIns(4,5)P_2. Another confusing theory is the fact that neither PtdIns(4,5)P_2 nor PtdIns(3,4,5)P_3 concentrate or even localize within the ER, this therefore raises questions about the physiological function of SKIP's ER localization (Dong et al., 2018).

SKIP possesses a simple two-domain structure with an N-terminal 5-phosphatase domain followed by a C-terminal SKICH domain, without any reported transmembrane regions (Figure 7a). Interestingly, INP54, which is the closest SKIP homologue in yeast also localizes at the ER surface, denoting a highly conserved ER-associated role of SKIP. However, INP54 do not possess the SKICH domain as seen in SKIP and thus, this may explain why the localization of INP54 to the ER is via a hydrophobic 13-aa C-terminal sequence which is lacked by SKIP (Dong et al., 2018).

SKIP is highly expressed in the brain, eye, kidney, and muscle (Ijuin et al., 2000). A number of genetic studies have linked SKIP's activity to congenital muscular dystrophy syndrome with short stature, cataracts, and mild intellectual disability (Osborn et al., 2017; Wiessner et al., 2017). However, the true mechanisms of this disease are not clear yet. Particularly, the presence of a SKIP pool which are not ER associated makes it difficult to deduce whether the ER localization of SKIP drives or participates in the disease

development (Osborn et al., 2017; Wiessner et al., 2017). Majority of reported mutations of the SKIP gene are missense mutations that frequently presents at the amino terminal, thus compromising SKIP's catalytic activity (Osborn et al., 2017). Just as other 5-phosphatases, SKIP expression inhibits PtdIns(3,4,5)P_3 signaling and Akt activation, particularly, in insulin-responsive cells. However, the specific function of SKIP in the regulation of cancer cell proliferation and migration is unknown (Davies et al., 2015). There is some evidence that SKIP shows altered expression in brain cancer (Bredel et al., 2005).

In cancer cells, SKIP has been shown to contribute to the regulation of cell migration, polarity, adhesion, and cell invasion (Bredel et al., 2005; Davies et al., 2015; Ramos et al., 2018). A recent study reported that the depletion of SKIP regulates cell migration in glioblastoma cells and this is dependent on integrin-mediated stimulation of PtdIns(4,5)P_2 leading to its abundance (Ramos et al., 2018). In the context of glioblastoma, SKIP is observed to be both decreased and increased in PTEN deleted glioblastomas (Davies et al., 2015). Consistently, a large-scale microarrays study using glioblastoma patient samples showed an increased or decreased expression of SKIP mRNA or DNA relative to normal control tissues (Bredel et al., 2005).

Overexpressing SKIP in PTEN null U87MG glioma cells decreased anchorage-independent cell growth and growth factor-induced PtdIns(3,4,5)P_3/Akt signaling. Even though, silencing SKIP expression did not impact cell proliferation, it induced a marked reduction in cell migration while increasing PtdIns(4,5)P_2 expression and decreasing activation of the actin-regulatory protein cofilin, which is also a PtdIns(4,5)P_2-binding protein (Davies et al., 2015). Of note, overexpressing SKIP in U87MG cells also suppressed migration at a similar rate as demonstrated during PTEN reconstitution, however, this is via separate mechanisms. It was shown that PTEN reconstitution was needed for lamellipodia formation and focal adhesion. Conversely, SKIP overexpression inhibited lamellipodia generation, talin incorporation into focal adhesions and the plasma membrane recruitment of PtdIns(4,5)P_2-interacting proteins (Davies et al., 2015).

Moreover, SKIP's activity represents one of the potent negative regulators of PI3K, which is an essential oncogenic pathway (Hedberg Oldfors et al., 2015). It has also been shown

that low SKIP expression in HCC patients correlates with poor clinical outcomes and that SKIP plays significant role in reducing overall survival and relapse-free survival. Thus, the authors recommend that SKIP mRNA expression may be an independent prognostic factor for HCC patients (Wang et al., 2020).

3. Brain tumors

Despite the fact that neural cell division is majorly restricted to the developmental period (during embryogenesis and early years after birth), the central nervous system (CNS) can be hijacked to support oncogenic events characterized by increased neural cell divisions at any point in time. These events can disrupt the intrinsic mechanisms that regulate cell division and DNA duplication, generating aberrant copies. As one of the major causes of human deaths worldwide, cancer represents a real challenge to the world (Goodenberger and Jenkins, 2012; Ostrom et al., 2018) (Figure 8A). Comparatively, CNS associated tumors or cancers are rare, representing about 1.4% of reported cancer cases in 2017, which equals an annual incidence of 6.4 cases per million worldwide. However, CNS tumors are among the deadliest forms of human tumors with poor survival rate and prognosis. Only about 33.6% of patients survive after five years, following diagnosis (Figure 8B) (Ostrom et al., 2018).

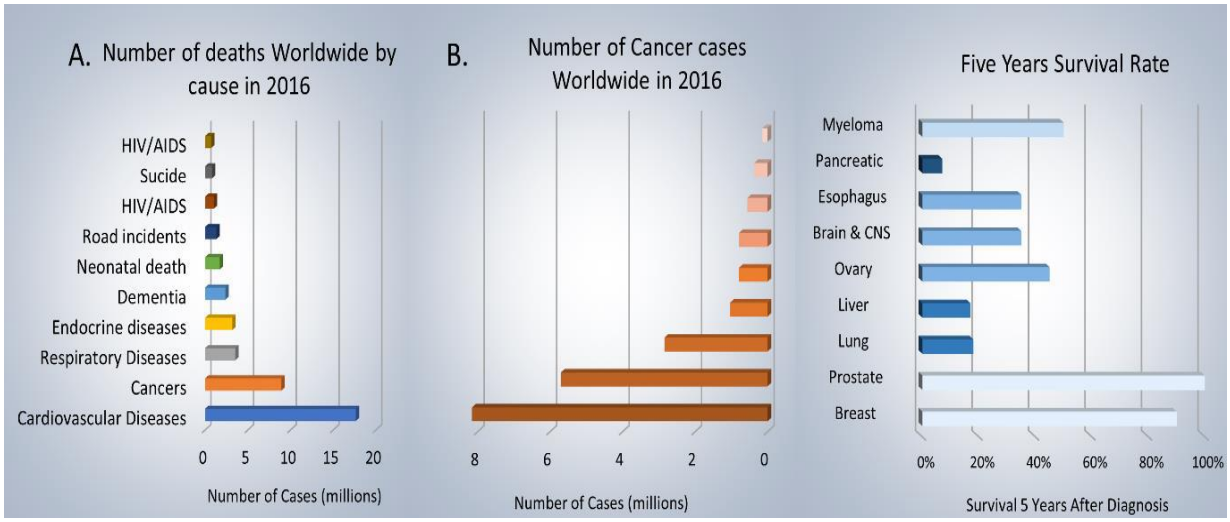


Figure 8. *Epidemiology of brain tumors and other cancers worldwide: (A) a representation of the causes of human deaths in 2016 (B) The number of cases per million for up to nine most deadliest cancers in 2016 (left) and their correlating five-year survival rate in percentage (right). Image adapted from* (Ostrom et al., 2018)

Brain tumors are mainly classified as either primary or secondary. Primary brain tumors generate "*de novo*" accounting for less than 5% of all tumors whereas secondary brain tumors which arise from the spread of malignant tumors generated elsewhere in the brain or body account for 30% of all tumors (Goodenberger and Jenkins, 2012; Ostrom et al., 2018). On the other hand, primary brain neoplasms can also be differentiated into classes depending on their histological characteristics, the origin of tumor cells, and their aggressiveness. Reported origins of primary brain tumors encompasses the embryonic, lymphatic, hematopoietic, meningeal or neuroepithelial tissues (Louis et al., 2016).

Gliomas belong to the family of neuroepithelial tumors which possess histological features like glial cells. The first classification of gliomas was made by the world health organization (WHO) where gliomas were classified into grades (I to IV) based on their aggressiveness. However, in 2016 WHO proposed an updated version of this classification which was defined by both histology and molecular features, thereby paving the for enhanced clinical, experimental, and epidemiological studies (Louis et al., 2016).

3.1 Glioblastoma

Glioblastoma (GBM) is one of the deadliest and most aggressive form of brain tumor characterized by a very poor prognosis as well as a median survival of approximately 15 months. Unfortunately, current GBM treatment therapies are not effective in treating GBM patients, only 3-5% survive up to 3 years or more (Louis et al., 2016; Lu et al., 2016). GBM histology is mainly displays necrosis and endothelial cell proliferation. Indeed, it has been shown that GBM possesses a high intra- and inter-individual heterogeneity and systematic recurrences which adds to the complexities in eradicating the disease (Adamson et al., 2009). Apart from Turcot's syndrome and Li–Fraumeni syndrome, most GBM patients develop GBM in a sporadic manner devoid of any recognized predisposing factors. Hence, little is known about the risk factors associated with the disease (Tian et al., 2018). Perhaps, looking at its epidemiology and etiology may provide further clarifications of the problem.

3.1.1 Epidemiology of Glioblastoma

Comparing GBM to other CNS tumors, GBM represents 15% of all malignant primary brain tumors as well as 56% of all reported gliomas (Figure 9A and B). GBM is frequent in patients between the ages of 55 and 60 years old, with 0.5 per 100.000 for children and 15.03 per

100.000 for patients aged between 74 and 84 years (Ostrom et al., 2018). Moreover, it has been observed that ethnicity and gender also affect the risk of developing GBM. For instance, non-Hispanic white Americans have higher risk of developing GBM compared to other ethnic groups (Ostrom et al., 2018) and 1.6 fold more men are likely to develop GBM than women (Tian et al., 2018).

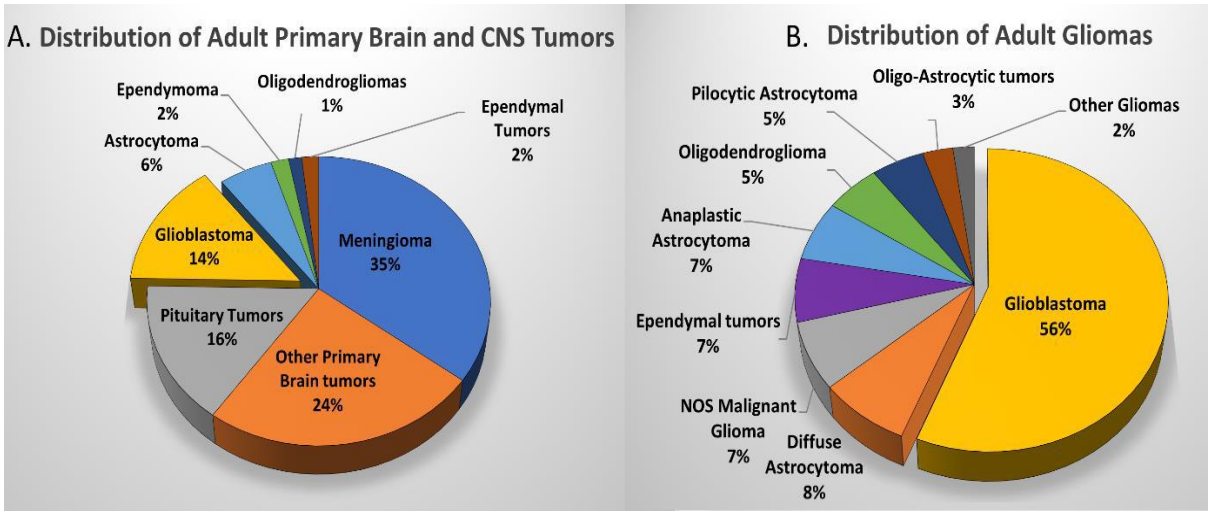


Figure 9: *Epidemiology of Brain Tumors and Glioma: (a) Glioblastoma accounts for 14% of all primary brain tumors in the United State of America. (b) Glioblastoma accounts for about 56% of all reported gliomas in the United States of America (Ostrom et al., 2018).*

3.1.2 Glioblastoma Classification

In general, brain tumors are grouped according to their histological resemblance and grade recommended by WHO. Histological studies provided the fundamentals of our current classification, however, molecular, and genetic parameters have been introduced recently and this brings our knowledge of grouping brain tumors to a higher level (Louis et al., 2016).

a. Histology/pathology

For decades, diagnosis of GBM relied on histology, proving to be a powerful tool for prognosis and therapeutic management. As mentioned previously, the first distinction among tumors can be identified based on the tumor origin, thus, helping to define the metastatic status of the tumor. Secondly, the affected tissue origin may also aid in distinguishing the tumors according to the histological subtype proposed by WHO (Louis et al., 2016). On this note, dependent on the tissue invasion of the tumor into the brain parenchyma, gliomas can be distinguished according to their grades. For example, lower grade glioma (i.e. grades I and II)

display a more circumscribed growth pattern than most high-grade cases (i.e. grade III and IV) which are mostly highly diffused and infiltrative. Tissue samples from high grade gliomas often display tumor cells aggregation near neuronal extensions, blood vessels or under the pial membrane, and are phenomenal characteristics observed in GBM (Adamson et al., 2009; Louis et al., 2016).

Generally, microvascular proliferation, nuclear atypia and the presence of necrosis are the predominant features used in the first line of GBM diagnosis (Figure 10). Morphological abnormalities and dysfunctional vasculature with microvascular proliferations, such as glomeruloid bodies are prominent features in GBM. The GBM morphology is comprised of multi-layered microvessels surrounded by hypertrophy and hyperplasia of endothelial cells, pericytes and smooth muscle cells, thus displaying a thick basement membrane in a wall of proliferating microvessels (Hardee and Zagzag, 2012) (Figure 10A). GBM is also frequently characterized by a higher mitotic activity, abnormal nuclear morphology and high cellular density (Stoyanov et al., 2017) (Figure 10B). However, these criteria are not enough for diagnosing GBM because of the similarities seen also in low-grade oligodendroglioma. Conversely to lower grade gliomas, which display extensive calcification, necrotic areas are more often prominent in high-grade gliomas like GBM. They consist of irregular, serpiginous foci circumscribed by densely compacted tumor cells (pseudopalisades) (Figure 10C) (Raza et al., 2002).

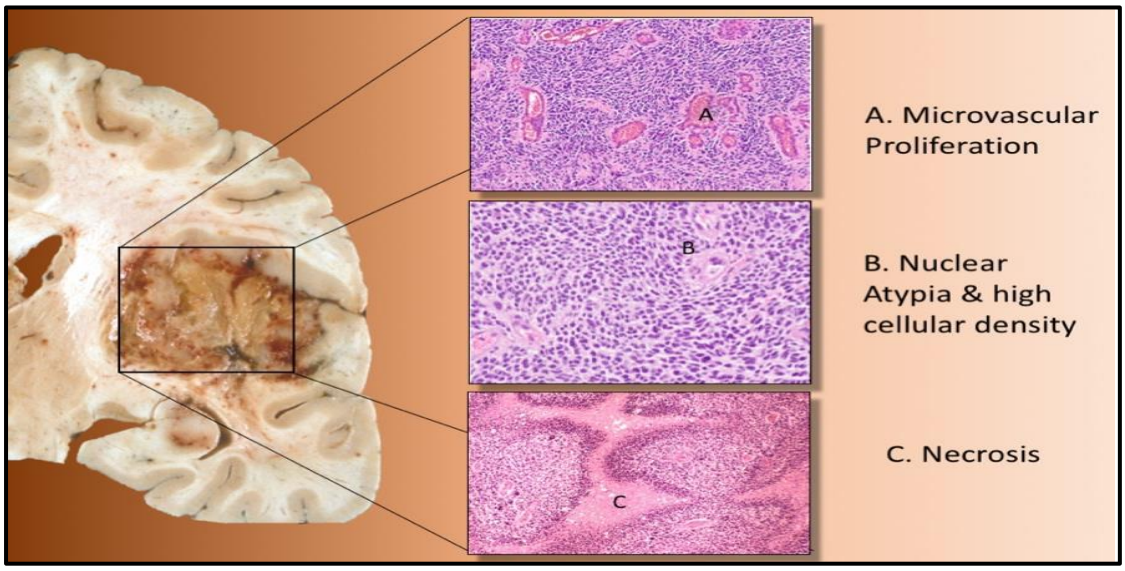


Figure 10*: Major histological features observed in GBM. Coronal portion of a brain slice from a GBM patient on the left with (A) increased microvascular proliferations and endothelial*

hyperplasia (B) Nuclear Atypia and high cellular density (C) Immunohistochemistry showing a necrosis plate surrounded by pseudopalisade cells (Ostrom et al., 2018; Raza et al., 2002; Stoyanov et al., 2017).

b. Biomarkers and Molecular Classification

To support or solve the issues that surround histological means of diagnosing gliomas, the recommendation by WHO on gliomas now considers genetic variants. The identification of an R132H mutation in *Isocitrate DeHydrogenase* (IDH-R132H) gene was considered a breakthrough discovery in glioma and GBM which gave rise to the first major change in their respective classifications (Louis et al., 2016; Ohgaki and Kleihues, 2013). While *IDH* mutation is observed in less than 10% of GBM cases and mostly in young adults, this mutation helps to define the glioma subgroups, especially when a significant number of secondary GBM cases develop from pre-existing diffuse or anaplastic astrocytomas (Figure 11) (Hartmann et al., 2010). Wild Type (WT) *IDH* GBM, comprising of more than 90% of all GBM, corresponds with primary glioblastoma occurring *de novo* in elderly patients without pre-existing lower grade tumors (Ohgaki and Kleihues, 2013). The differences between these two groups is not only of biological importance but it also allows the award of clinical features like the mean age of onset or patients overall survival (Hartmann et al., 2010). On the other hand, another important genetic feature is the status of *ATRX* gene, which is lost in astrocytoma, in opposition to 1p/19q co-deletion as observed in oligodendroglioma (Figure 11). Interestingly, the *ATRX* gene loss is also present in GBM *IDH* mutant (Nandakumar et al., 2017).

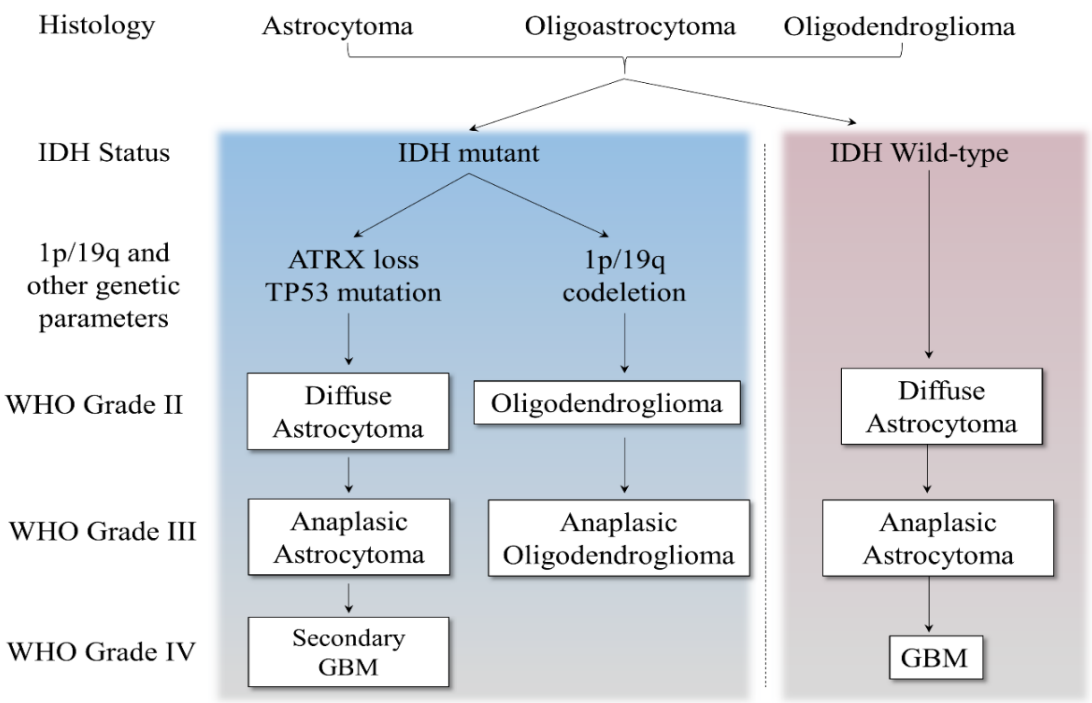


***Figure 11**: New WHO recommended Decision Tree for CNS tumor classification**. This is representative scheme of the new WHO recommended decision tree which considers genetic markers like IDH mutation, loss of ATRX, TP53 or TERT mutations and co-deletion of 1p/19q chromosome as part of the definition of CNS tumor grades and subtypes. Image adapted from* (Nandakumar et al., 2017).

The development of a large-scale molecular profiling study a couple of years ago aided in the definition of the different GBM subtypes. This study grouped GBM into four distinct subtypes based on the gene expression profile of GBM patients, accessible on TCGA, namely: neural, proneural, classical and mesenchymal (Verhaak et al., 2010). More importantly, the molecular classification of GBM subtypes based on their methylation profiles was demonstrated to be more robust and reliable, reflecting the cells of origin and displaying phenotypes which remain stable even during tumor evolution (Lee et al., 2015). Among these subgroups, the mesenchymal subtype has been shown to possess stronger resistance to radiotherapy as well as shorter survival periods (Olmez et al., 2018). The aggressiveness demonstrated by this subtype is mainly attributed to high proliferation of cells, enhanced migratory and invasiveness of cells and an increased resistance to current treatments (Olmez et al., 2018).

The new GBM classification encompasses all these molecular profiles to separate GBM cases into eight different subgroups (Louis et al., 2016). Firstly, there are four GBM subtypes attributed to children based on the type of mutations occurring on two genes: *BRAF* and *H3F3A*. In young adults, two GBM subtypes are defined based upon mutations of the *IDH* gene or at the promoter of Telomerase Reverse Transcriptase (*TERT)* (RTK I) and this is often characterized by a proneural gene expression profile (Figure 12). For adults, the first GBM subtype (RTK II) is characterized by epidermal growth factor receptor (*EGFR)* amplifications whereas the second group (Mesenchymal) exhibits mutations in *NF1* and a mesenchymal gene expression profile (Brennan et al., 2013; Lee et al., 2015; Louis et al., 2016).

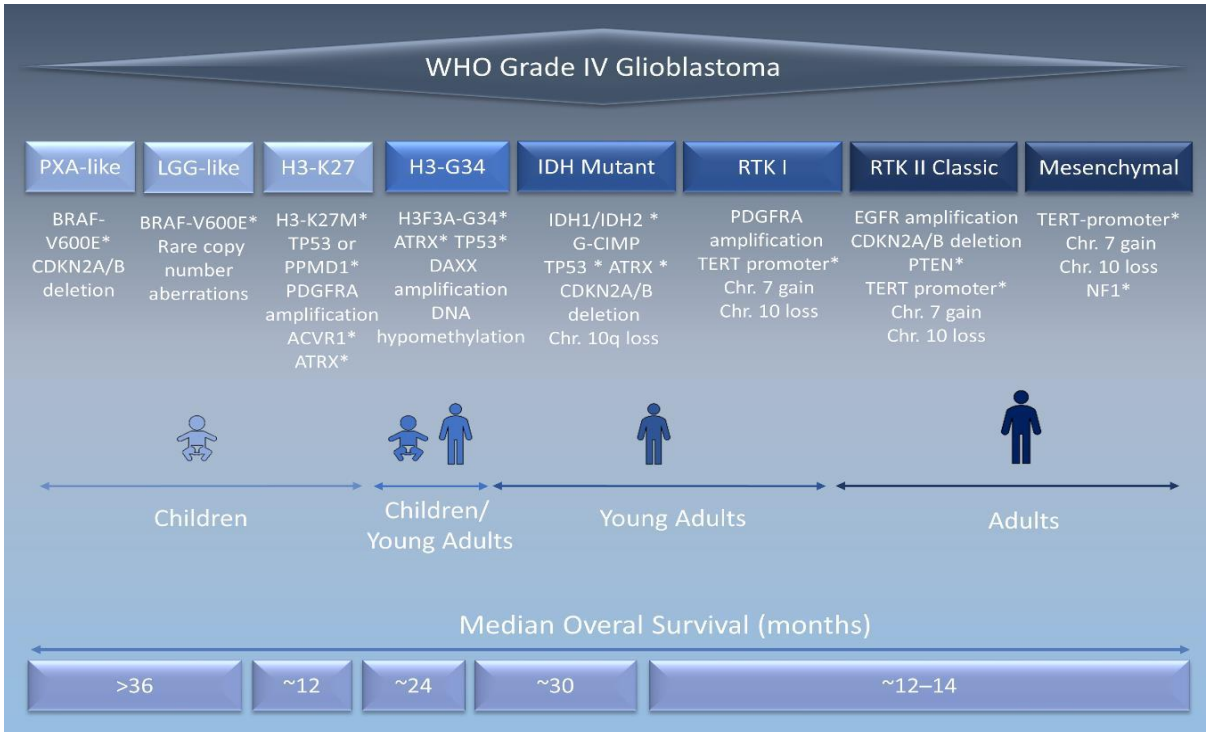


Figure 12: Classification of GBM based on molecular and genetic variations. *This is a representative scheme that defines the criteria for identifying or classifying GBM into the various subtypes. The upper row defines the different subtypes with their various genetic characteristics showing just below them. The different colorings of each group represents the general age of GBM onset. At the bottom, the median survival period of each GBM subtype demonstrates the pathogenicity of the tumor type (* =Mutations)* (Louis et al., 2016).

AIM OF THE STUDY

This study aimed at determining the pathological impact of PI metabolic enzymes, PLCβ1 and SKIP, in GBM patient samples as well as GBM cell line models.

So far, most studies of PLCβ1 in GBM are done in silico, hence it is imperative that the impact of PLCβ1 in the regulation of cellular mechanisms evidenced in GBM is studied at the molecular level. As such, human clinical samples were used to evaluate the gene expression levels of PLCβ1 and other PLCs in GBM, oligodendroglioma, and astrocytoma low-grade gliomas.

GBM cell line models were then used to study the direct impact of altered PLCβ1 gene and protein expression on cellular mechanisms that become aberrant in GBM such as:

- Cell adhesion and migration
- EMT
- Cell survival
- Expression of PtdIns(4,5)P_2 at the plasma membrane.

In addition, cellular and mouse models of GBM were exploited to study the impact of SKIP in GBM pathogenesis. Molecular and genetic tools were employed to downregulate SKIP expression in GBM cell line models in order to study:

- SKIP impact on nuclear PtdIns(4,5)P_2 and nuclear speckle marker SC-35
- The effect of SKIP in tumor formation in xenograft mice
- SKIP impact on apoptosis, proliferation, and viability

Materials and Methods

1. Materials

Anti PLCβ1 (PA5-78418), Anti phospho-FAK Tyr 397 (Lot no. 2099989), and Anti-FAK (Lot no. TC268043) were obtained from Invitrogen (Milan, Italy). Anti-Slug (SC 166476) was obtained from Santa Cruz (Milan, Italy). Anti-Twist1 (Cst 46702S), anti-vimentin (Cst D2H13), anti-pAKT (Cst 4058), anti-Akt (Cst 9272), anti-mTOR (Cst 2974), anti-total mTOR (Cst 2983), anti-Phospho ERK 44/42 (Cst 4376) were all obtained from cell signaling (Milan, Italy). Anti-SKIP rabbit polyclonal antibody was from LifeSpan BioSciences, Inc (BioConnect, The Netherlands; cat.no. LS-B10239). Anti-PtdIns(4,5)P_2 (2C11) (cat.no. Z-P045) antibody was from Echelon Biosciences, Inc (Milan, Italy). Mouse anti–SC–35 (cat.no. 556363) was from BD Biosciences (Erembodegem, Belgium). Normal horse serum (NHS) (cat.no. 26050–070), Alexa-Fluor-488-conjugated donkey (cat.no. A21202), Alexa-Fluor-594-conjugated goat (cat.no. A11012) anti-rabbit-IgG, Alexa-Fluor-488-conjugated donkey (cat.no. A21206) and Alexa-Fluor-594-conjugated goat (cat.no. A11032) anti-mouse-IgG, Hoechst 33342 (cat.no. H-3570) were from Invitrogen (Breda, The Netherlands). Mounting medium (cat.no. C0563) was from Dako (Heverlee, Belgium). Rhodamine-Phalloidin and Fibronectin (cat.no. F1141) were obtained from Sigma-Aldrich (Milan, Italy).

2. Cell culture and passaging

Human GBM cell lines U87 MG and U-251 MG and human primary GBM cells GB138 cells were cultured to 70% confluence in 10% serum containing media with 1% penicillin-streptomycin and maintained at 37 °C, 5% CO_2. Culture media were taken out of flasks using pipette and cells were washed with 1× PBS with pH of 7.4. After rinsing with PBS, cells were detached with 0.25% (v/v) of Trypsin in 2mM EDTA. Flasks were kept in a humidified incubator set at 37°C with 5% CO_2 for 3-5 minutes in order to allow detachment of adherent cells from flasks. Equal volumes of fresh media containing serum were used to neutralize trypsin before centrifuging at 300g for 5 minutes. Cell pellets formed were re-suspended in fresh complete medium and seeded back into flasks.

3. PLCβ1 knockdown via lentiviral transduction

HSHO96803-LVRHIGP Homo Sapiens PLCβ1 for knockdown (PLCβ1 KD) and CSHCTR001-LVRHIGP empty control vector plasmids (SHX control) from Genecopoeia, USA were used in the lentiviral construct to knockdown PLCβ1 in cell lines and their controls respectively. The Lenti-Pac HIV expression packaging kit also from Genecopoeia, USA was used as the viral packaging unit for transfecting HEK293T cells (ATCC, USA) according to manufacturer's protocol. After lentivirus transfection, cells were selected with puromycin for at least 3 weeks to form stable clones.

4. U-87 MG cells xenografts

All the methods involving mice models for xenograft tumor growth assays were approved by the Animal Care and Ethics Committee of the Université Libre de Bruxelles. Eight weeks old female, athymic immunodeficient NOD/SCID mice were obtained from Charles River Laboratories (France). 1×10^6 cells were suspended in 50 µl DMEM + 50% growth factor-reduced extracellular matrix and injected in duplicates subcutaneously in 4 mice per cell line. The mice were sacrificed to collect tumors formed after 5 weeks post injection for tumor volume and weight analysis. The tumor volume was measured using the formula length × $(\text{width})^2/2$.

5. Cell viability assay

Cell viability was determined using the CellTiter-Glo® One Solution Assay (Promega, Milan, Italy) according to the manufacturer's instructions. 5000 cells per well were plated in 96-well plates, in triplicates for each condition, and incubated overnight in 100µl of complete media. To measure cell viability at time points 0, 24 and 48 h, 20 µl of cell viability reagent was added to each well and cells were incubated at 37 °C for 2-4 hours. The optical density was determined at 490 nm and compared to a blank made without cells.

6. BrdU cell proliferation assay

In order to measure cell proliferation in SKIP depleted cell, 20,000 cells were seeded on glass cover slips in 24 well plates for 24 h. 100 µM of 5-bromo-2'-deoxyuridine (BrdU) and 4 µM of 5-fluoro-2'-deoxyuridine (FldU), both from Sigma-Aldrich, were added to the cells before 1 h fixation with methanol. DNA incorporated with BrdU was immunodetected as prescribed previously (Roger et al., 1992). Hoechst was used to stain the nucleus and the ratio of BrdU

positive cells to total cell count was analysed in a random manner. This is represented as a percentage.

7. Total RNA extraction

The RNeasy Mini Kit (Qiagen, Milan, Italy) was used to extract total RNA from growing cells with ≥ 80% confluency according to the manufacturer's protocol. The RecoverAll™ Total Nucleic Acid Isolation Kit (Invitrogen, ThermoFisher Scientific, Monza, Italy) and the RNeasy Lipid Tissue Mini Kit (Qiagen, Milan, Italy) were used to extract total nucleic acids from FFPE and fresh frozen clinical tissues, respectively while following manufacturer's protocol. The nanodrop spectrophotometer was used to quantify the extracted total RNA. With the help of the Qubit fluorometer (Invitrogen, ThermoFisher Scientific, Monza, Italy) and its recommended assay, the Qubit RNA IQ Assay, total RNA from FFPE samples were analysed for their quality before use. Only samples with Qubit IQ number of 7 or greater were selected for further experiments. Total RNA samples were aliquoted and stored at -80 °C for subsequent experimentations.

8. **Reverse transcription and real-time PCR**

Using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, ThermoFisher Scientific, Monza, Italy) 1 µg of total RNA were reverse transcribed into cDNA following the manufacturer's protocol. With TaqMan Universal PCR Master Mix (ThermoFisher Scientific, Monza, Italy), mRNA expression levels were detected using a TaqMan probe-based real-time PCR system. Real-time PCR was performed with the ABI PRISM 7300 real-time PCR machine (Applied Biosystems, Life Technologies, Monza, Italy). β-actin was used as the housekeeping gene, data are presented as fold changes relative to the expression levels of control samples in accordance with the $2^{-\Delta\Delta CT}$ formula. Validated PLCβ1 gene probe (Hs.PT.58.24665550) was used.

9. Whole cell lysates or protein extraction

Cell pellets were washed twice with Ice cold 1× PBS pH 7.4 before lysing cells with T-PER protein lysis buffer supplemented with Halt protease and phosphatase inhibitor cocktails (all from ThermoFisher, Monza, Italy). Cells were vigorously shaken on ice for 30 minutes and sonicated in a cycle with 15 seconds duration and at a power of 40-50% before centrifuging

at ≥ 1200RPM for 15 minutes at 4°C. Resulting supernatant or whole cell lysates were transferred into a new tube for protein quantification using the Bradford Protein Assay (Bio-Rad, Milan, Italy).

10. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

To maintain consistency, all protein homogenates were separated with Bolt 4-12% polyacrylamide-0.1% commercial SDS gels (ThermoFisher, Monza, Italy). Wells were always washed with 1× running bolt before protein homogenates (40 µg -50 µg) were loaded. Before loading of the proteins, calculated volumes of Loading buffer and reducing buffer are added to the required volume of proteins. This mixture was then heated for 5 minutes at 96°C. A voltage of 90mA was conducted to allow the protein homogenates to run through the stacking gel. The voltage was later increased to 120mA to allow the sample to separate in the resolving gel.

After resolving proteins with SDS PAGE, the proteins were then transferred from the gel onto a nitrocellulose membrane soaked in a pre-chilled 1× transfer buffer. In transferring, the gel and membrane were packaged as a sandwich placed between cushions and then fitted in a cassette. A roller was rolled on the surface of the sandwich to prevent bubbles from interfering with the transfer. The cassette was then placed in a transfer rack together with an ice pack positioned beside it. Pre-chilled 1×transfer buffer was then poured to fill the transfer setup containing the rack. A current of 100A was passed through the setup for 1 hour at 4^{o}C. After transferring onto membrane, membranes were washed 3 times with 1× PBST pH 7.4 at 10 minutes interval and then blocked with blocking buffer (5% non-fat dry milk in PBST) for 1 hour. Membranes were incubated with primary antibodies overnight at 4°C on a rocking stirrer. Membranes were washed again 3 times with 1× PBST pH 7.4 at every 10 minutes and then incubated with peroxidase conjugated secondary antibodies (ThermoFisher Scientific, Monza, Italy) diluted in PBST for 1 hour at room temperature. ECL enhanced chemiluminescence reagents (ThermoFisher Scientific, Monza, Italy) were used to detect immunoreactive bands and images captured with the ChemiDoc Gel Docking system (Bio-Rad, Milan, Italy).

11. Membrane Striping

To dispose of the attached primary and secondary antibodies, membranes were stripped to either check for other proteins or assess the quantity of proteins loaded onto the gels. Membranes were placed in a solution containing 50ml of stripping buffer and 365µl of 2-β-mercaptoethanol (Sigma Aldrich); this was incubated for 30 minutes in a heating chamber set at 50^{o}C. Membrane was then transferred into 1× TBST, pH 7.4 for washing; it was washed 3 times at 10 minutes intervals and then blocked with blocking buffer for 1 hour. The process continues as described in the western blotting procedure; different antibodies were used to detect different target proteins.

12. Immunofluorescence

Cells were fixed in 4% paraformaldehyde/phosphate buffer saline (PBS) for 20 min at 4 °C. They were then permeabilized with 0.3% Triton X-100 in PBS for 5 min at 4 °C. After blocking in 1% bovine serum albumin (BSA) for 1h at room temperature, cells were incubated with primary antibody overnight at 4 °C. For detection of plasma membrane PtdIns(4,5)P_2, cells were fixed with 3.7% paraformaldehyde, 0.3% gluteraldehyde and 0.01% saponin. Cells were then blocked with 1% BSA and 1% goat serum in PBS. Dilution of primary antibodies were in accordance with the manufacturer's instructions, usually overnight at 4 degrees. After a series of washes with 1x PBST, cells were then incubated in the dark at room temperature for 1 hour with corresponding secondary antibodies, Anti-Mouse IgG F(ab')2 Fragment antibody conjugated to Alexa Fluor 488 (Cell Signaling Technology, Leiden, The Netherlands) or Anti-Rabbit IgG F(ab')2 fragment-Cy3 antibody. Lastly, nuclei were stained with ProLong Gold Antifade reagent with DAPI (Invitrogen, Thermo Fisher Scientific, Monza, Italy). Slides were then examined under a Zeiss Axio-Imager Z1 fluorescent microscope (Carl Zeiss International., Germany).

13. Quantification of nuclear PtdIns(4,5)P_2, SC-35 and SKIP

The method used was adapted from (Ramos et al., 2018). Measurement of nuclear staining signal was conducted on cells showing similar Hoechst staining, using Image J. A maximum intensity projection was created and after splitting the channels, Hoechst staining was used as a threshold mask in the selection of the nuclei to be quantified. Based on the mask, the corrected total cell fluorescence (CTCF) were calculated for nuclear PtdIns(4,5)P_2, SC-35 and

SKIP. However, SC-35 staining was used as a threshold mask in some cases, especially in nuclear speckles to evaluate the CTCF of PtdIns(4,5)P_2 or SKIP.

14. Cell Adhesion Assay

Cultured cells at 80% confluence were serum starved in starvation media without FBS overnight before allowing to spread on 5ng/µl of Fibronectin and 0.1% BSA as control for 2 hours in a 96 well plate. Following subsequent washes in PBS to eliminate non-adherent cells, adherent cells were fixed with ice-cold ethanol for 10 minutes and stained with crystal violet solution for 30 minutes at room temperature. After several washes to wash off excess crystal violet stain, cells were then permeabilized with 0.2% Triton X-100 in PBS and absorbance at 590nm was read using the spectrophotometer.

15. Transwell Migration Assay

Chemotactic migration of cells was evaluated using the transwell assay. 40,000 cells in serum free media were seeded into transwell inserts of 8 µm size. Migration was potentiated by the addition of 10% FBS in minimum essential medium in the outer chambers of the inserts. Cells were allowed to migrate overnight. Non-migrated cells were removed with a cotton swab while migrated cells were fixed with 96% ice-cold methanol followed by staining with 0.1% (w/v) crystal violet. Images of stained cells representing migrated cells were then captured using a Nikon digital camera.

Statistics

Data are represented as mean ± SEM and were analyzed by GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Data analyses between two independent groups were performed using Student's t-test (two tailed) followed by Mann-Whitney and analysis of variance (ANOVA) to determine statistical significance among multiple independent groups, unless otherwise stated. Data were considered significant at 95% confidence level.

Results

1. In-silico analysis of PLCβ1 expression in GBM and low-grade glioma and its impact on patients' survival

As previously reported, PLCβ1 is a candidate signature gene for the proneural subtype of GBM and PLCB1 expression inversely correlated with pathological grades of glioma (Lu et al., 2016). To confirm these findings, we used a different analysis software. Data from the cancer genome atlas (TCGA) were extracted and analysed using the innovative online platform, GEPIA (Tang et al., 2017), to evaluate the expression levels of PLCβ1 mRNA in GBM, low grade glioma (LGG) and normal human tissues. Results showed consistency with previous findings, in that PLCβ1 mRNA levels are low in GBM samples compared with low grade glioma. In addition, a comparison was also made with the mRNA levels of PLCβ1 in normal human samples. The mRNA levels of PLCβ1 in GBM was significantly lower than in both normal and low-grade samples. However, there was not a vast difference in PLCβ1 expression between normal and low-grade samples. Regarding patients' survival, low PLCβ1 expression in GBM patients correlated with less survival even though without statistical significance. In low-grade glioma, high PLCβ1 expression significantly correlated with increased patients' survival (Figure 13).

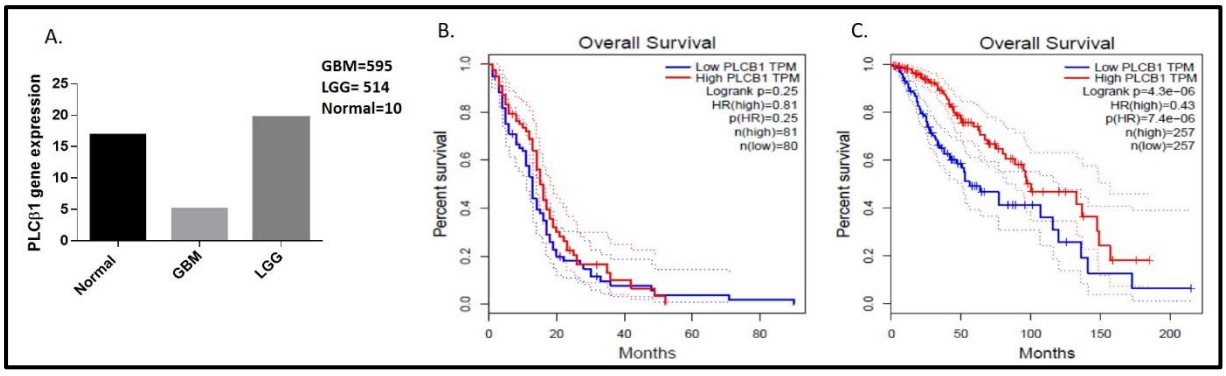


Figure 13. *GEPIA data analysis showing PLCβ1 gene expression and its implications on patients' survival. A. PLCβ1 gene expression in GBM clinical samples are lower compared with normal and low-grade glioma tissues. Analysis involved 595 GBM samples, 514 low-grade glioma samples consisting of both astrocytoma and oligodendroglioma patients' samples, and 10 normal human brain tissues. B. Kaplan-Meier curve showing that low GBM gene levels in GBM correlates with less survival of GBM patients. C. In low-grade samples, higher PLCβ1 levels correlate with higher survival of patients* (Tang et al., 2017).

2. PLCβ1 is downregulated in GBM and is inversely correlated with low-grade gliomas

Since the data obtained from a different analysis software confirmed that PLCβ1 gene was indeed downregulated in GBM and correlated with poor survival, the next step for this study was to analyse patients' samples. This experiment benefitted from a partnership between the Cell Signaling Laboratory of the University of Bologna and the Neurological department of the Bellaria hospital, all in Bologna. RNA extracted from both fresh frozen and formalin-fixed paraffin embedded (FFPE) clinical samples of GBM, astrocytoma and oligodendroglioma patients were analysed to evaluate the gene expression of PLCβ1 and other PLCs. Indeed, PLCβ1 expression in GBM tissues were downregulated compared to the levels observed in astrocytoma and oligodendroglioma tissues, thus confirming the findings from the in-silico study using GEPIA. PLCβ1 expression in both fresh frozen and FFPE samples remained consistent. Other PLCs, PLCβ4 and PLCδ3 were also downregulated in GBM compared to the low grades. However, when compared to healthy control samples, only PLCβ1 and its splice variants 1a and 1b were downregulated (Figure 14). This suggests that PLCβ1 gene expression truly correlates inversely with Glioma pathological grades, the higher the grade, the lower the expression.

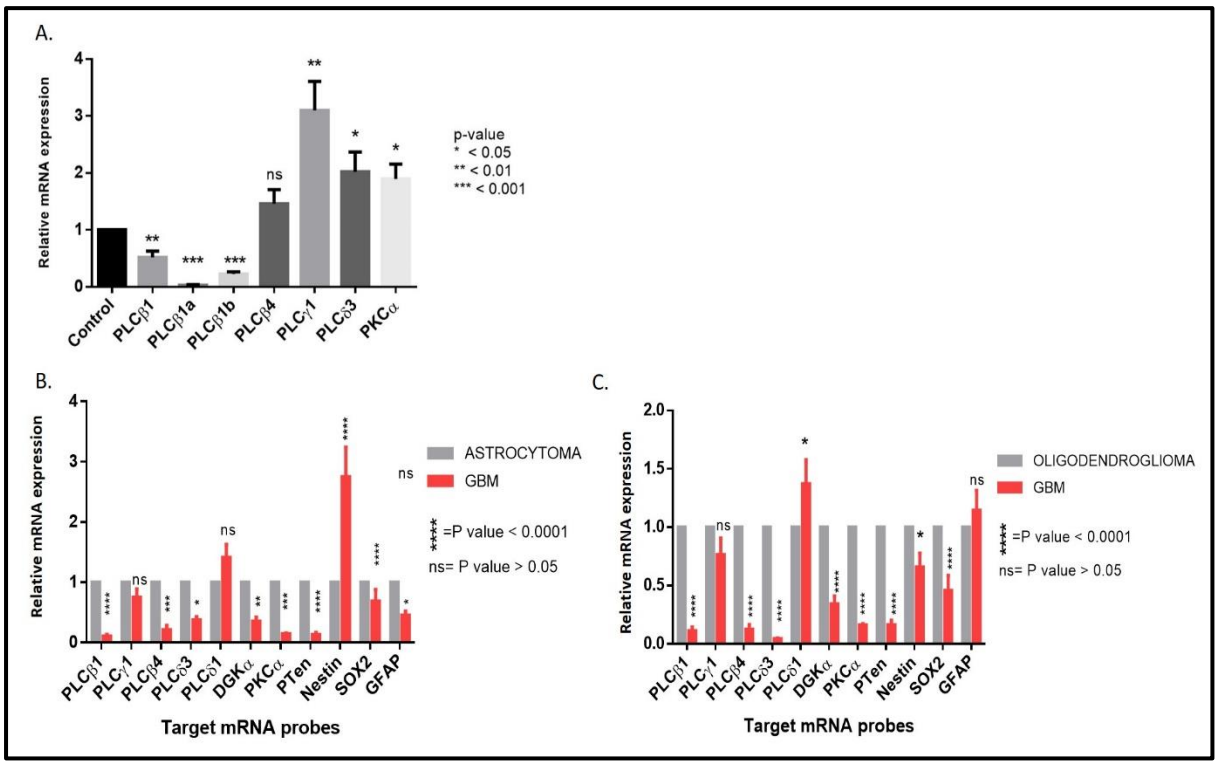


Figure 14. *PLC signaling network in GBM and low-grade glioma. A. qPCR analysis of the mRNA levels of selected PLC family members and their downstream target PKCα in fresh frozen tissues of GBM (n=20 samples) clinical samples and healthy brain controls (n=5 samples). B. Expression levels of the mRNA of selected PLC family members and their downstream targets in GBM tissues (n=5 samples) and Astrocytoma tissues (n=3 samples). C. GBM tissues (n=5 samples) and Oligodendroglioma tissues (n=4 samples). Error bars represent mean ± SEM, ** p< 0.01, *** p< 0.001, ns= non-significant.*

3. Phospholipase signaling in PLCβ1 knockdown U87MG glioblastoma cell line

The PLC signaling network was analysed in U87MG cell lines after a stable knockdown of PLCβ1 in the cells. There is somewhat a compensatory mechanism allowing the upregulation of other PLCs, such as PLCγ1 and PLCβ4 when PLCβ1 is knocked down in U87MG cells. It was also shown that the mRNA expression of both isoforms of PLCβ1 were decreased as the total form decreased (Figure 15).

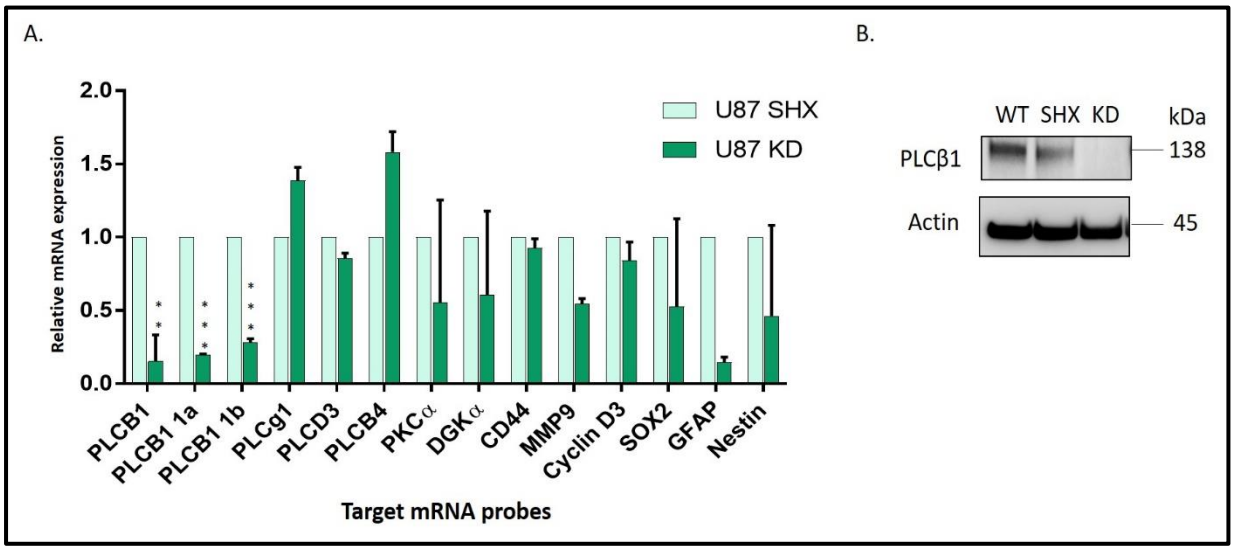


Figure 15: *Stable Knockdown of PLCβ1 in U87MG GBM cell line and PLCβ1 downstream effects. A. qPCR analysis of the mRNA expression of PLCβ1, its spliced variants, other PLCs and their potential downstream targets. B. Western blot analysis showing a significant knockdown of PLCβ1 in U87MG cells (from here on called KD) when compared with wildtype U87MG samples (here on called WT) and control samples with only the vector (from here on called SHX cells). Error bars represent mean ± SEM, ** p < 0.01, *** p < 0.001.*

4. PLCβ1 knockdown potentiates cell migration by promoting lamellipodia formation

Staining both PLCβ1 knockdown cells and empty control with the F-actin target, phalloidin, showed that PLCβ1 knockdown cells possessed lamellipodia protrusions as compared to more roundish membrane of empty vector control cells (Figure 16). Following this, a transwell migration assay was conducted to measure the migratory ability of the cells. Consistent with lamellipodia protrusions in PLCβ1 knockdown cells, it was shown that cell migration is also increased in these cells compared with empty vector control cells. Thus, supporting the role of PLCβ1 in glioblastoma cell migration.

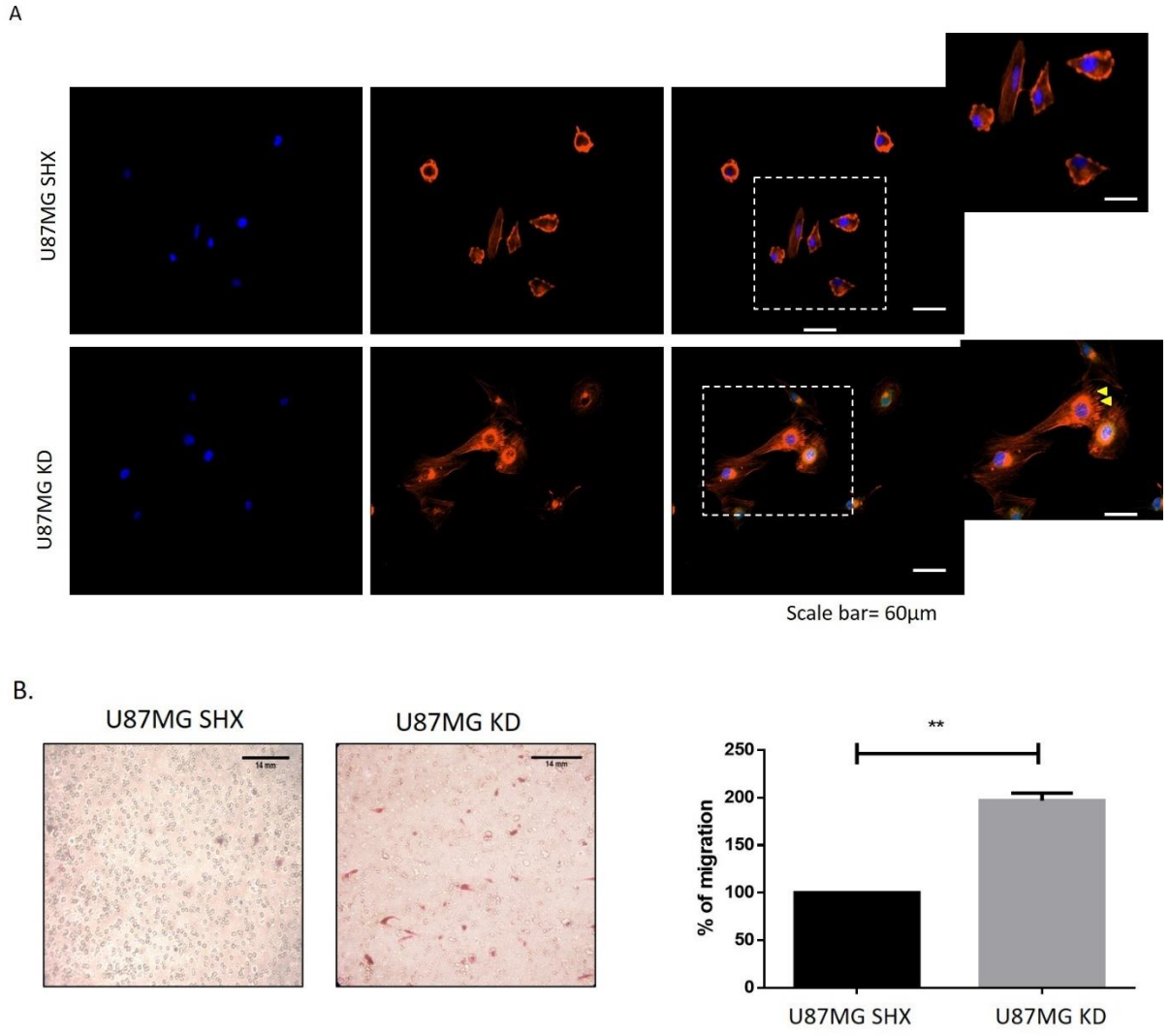


Figure 16. *PLCβ1 controls cell migration through actin dynamics. A. Cells were allowed to adhere and spread on 5µg/µl of fibronectin for two hours before fixation with 4% paraformaldehyde and followed by immunostaining. PLCβ1 KD cells show prominent lamellipodia formation (shown with yellow triangle symbols) as compared to more roundish control cells. Bar is set at 60 µm and bar for enlarged images are set at 10 µm. B. Image captures the violet colour produced by crystal violet staining of cells that migrated towards*

*the chemoattractant (media with 10% fetal bovine serum) in a transwell migration assay, suggesting that the PLCβ1 cells migrated more than wildtype cells and empty vector control cells. Images were captured with Nikon camera and a Zeiss light microscope at an overall magnification of x200. Scale is set for both images at 14 µm. Migrated cells counted using Image J to calculate percentage of migrating cells. Images from 4 different fields per condition and in duplicates were analyzed. Error bars represent mean ± SEM ** p = 0.0079.*

5. PLCβ1 affects cell-ECM adhesion in GBM by regulating focal adhesion kinase (FAK) activity

In U87MG cells with a stable PLCβ1 knockdown, the cells' adhesion to fibronectin, which is a component of ECM, was increased compared to control cells expressing PLCβ1. Cells were allowed to adhere on 5 µg/µl of Fibronectin at different time points of 30 minutes, 1 hour, and 2 hours. Interestingly, at both 1 hour and 2 hours, adhesion was higher in PLCβ1 knockdown cells (Figure 17A).

Since PLCβ1 knockdown in GBM cells promoted cell-ECM adhesion, it became important to also study the influence of PLCβ1 on the focal adhesion kinase (FAK). FAK regulates focal adhesion turnover and the cell spreading process. Employing immunofluorescence staining and Western blot techniques, the knockdown of PLCβ1 promoted the increase of FAK tyrosine activation (Tyr 397) (Figure 17).

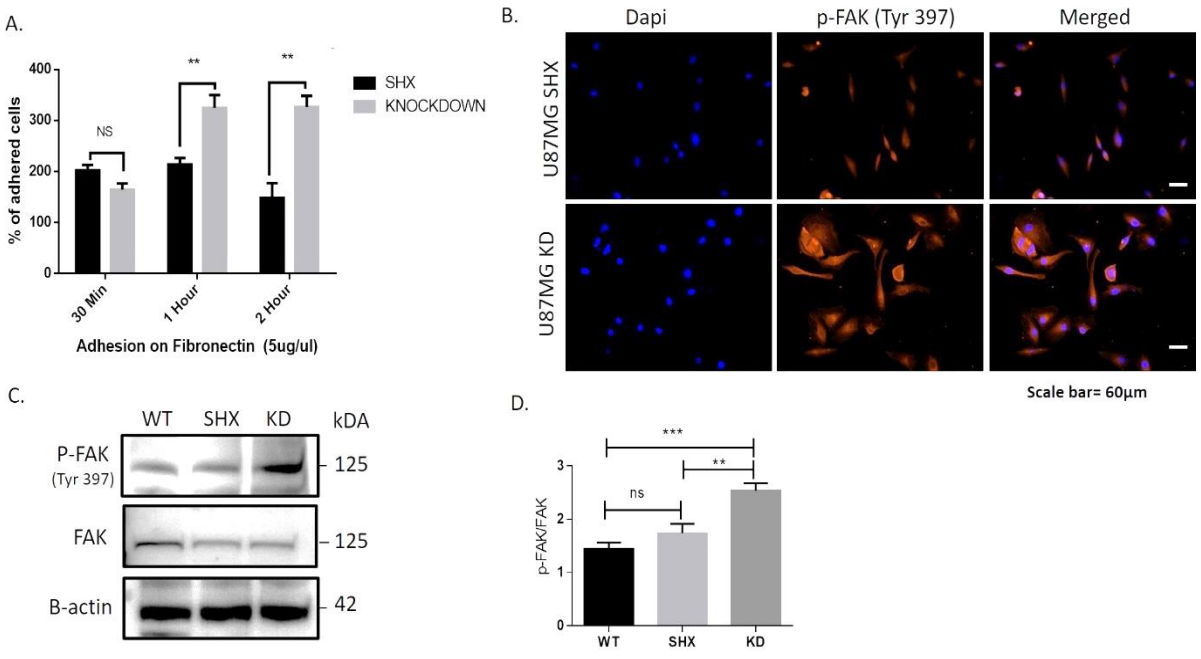


Figure 17. *PLCβ1 mediates cell-ECM adhesion through FAK activation. A. This graph represents U87MG cells that have been allowed to adhere on 5 µg/µl of fibronectin at different time points. B. Immunofluorescence staining showing high expression of p-FAK (Tyr 397) in PLCβ1 knockdown U87MG cells as compared to empty vector control. C. Western blot representation of the protein expression of p-FAK, total FAK and B-actin. D. This graph represents the quantification of p-FAK/total FAK, using Image J. Graphpad prism software was used in plotting data obtained from Image J. Non-parametric student's t-test was conducted to evaluate the statistical significance between two groups. ** p < 0.01, *** p < 0.001, ns: non-significant.*

6. PLCβ1 expression affects epithelial-mesenchymal transition (EMT)

In U87MG GBM cells, the knockdown of PLCβ1 promotes an increase in the protein expression of EMT transcription factors twist-1, and slug as well as an increase in vimentin which is a mesenchymal marker.

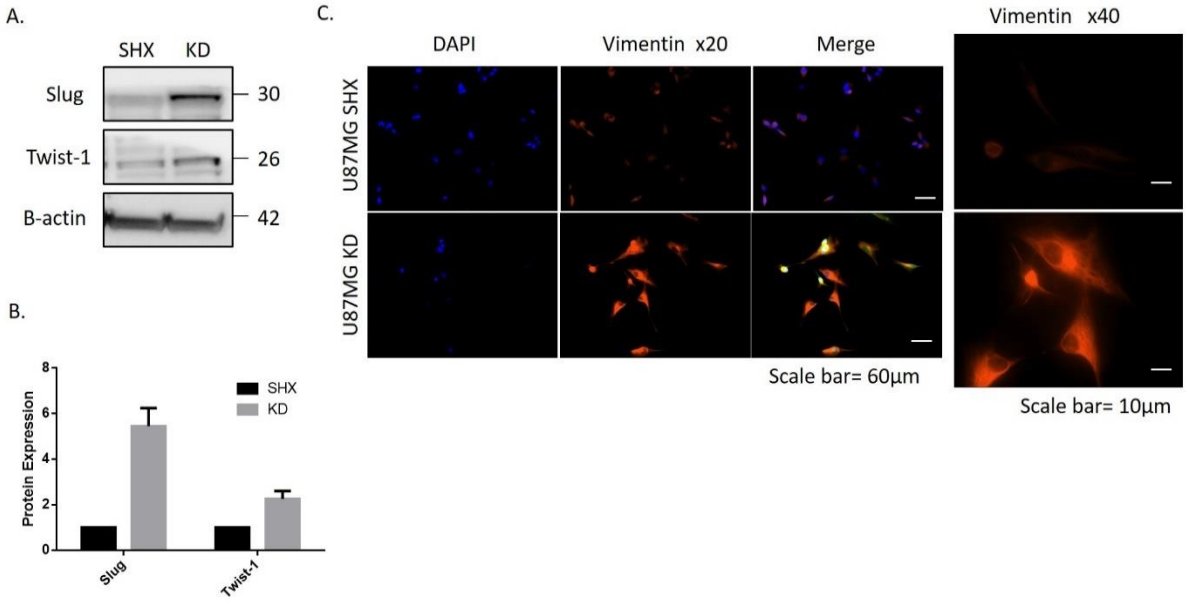


Figure 18. *PLCβ1 expression is involved in EMT. A. Western blot representation showing that PLCβ1 knockdown cells express increased transcriptional markers for mesenchymal phenotypes including Slug and Twist-1. B. A graph showing the quantification of expressed proteins in A, slug and twist-1 which are normalized to control SHX using image J analysis software. C. Both PLCβ1 knockdown U87MG cells and empty vector control cells were fixed with 4% paraformaldehyde for immunofluorescence staining. Dapi stained the nucleus blue and vimentin was stained in red.*

7. The effect of PLCβ1 in GBM cells in response to EGF

PLCβ1 knockdown cells and empty control cells were treated with 5 ng/µl of EGF at different time points up to 15 minutes to evaluate the effect of PLCβ1 on EGF-induced Akt activation. Other oncogenic survival regulators, like mTOR and ERK were also evaluated via Western blot. PLCβ1 knockdown in U87MG GBM cells inhibited EGF-induced Akt activation by decreasing the phosphorylation of Akt on serine 473; this was more evident at 10 and 15 minutes of EGF treatment. In addition, mTOR activation on the serine 2448 was also blocked in PLCβ1 knockdown cells, even at the earliest treatment time of 5 minutes (Figure 19). ERK activation was also modified in response to EGF. ERK phosphorylation was inhibited significantly in PLCβ1 knockdown cells when the cells were treated with EGF for 10 minutes. Most importantly, this was confirmed in the viability of cells using Promega one solution cell viability assay. Cultured cells were starved and treated with or without EGF to measure the effect on viability. Consistent with the expression of survival factors, pAKT and p-mTOR, viability was decreased in PLCβ1 knockdown cells with or without EGF when compared to scrambled cells (Figure 19 D and E).

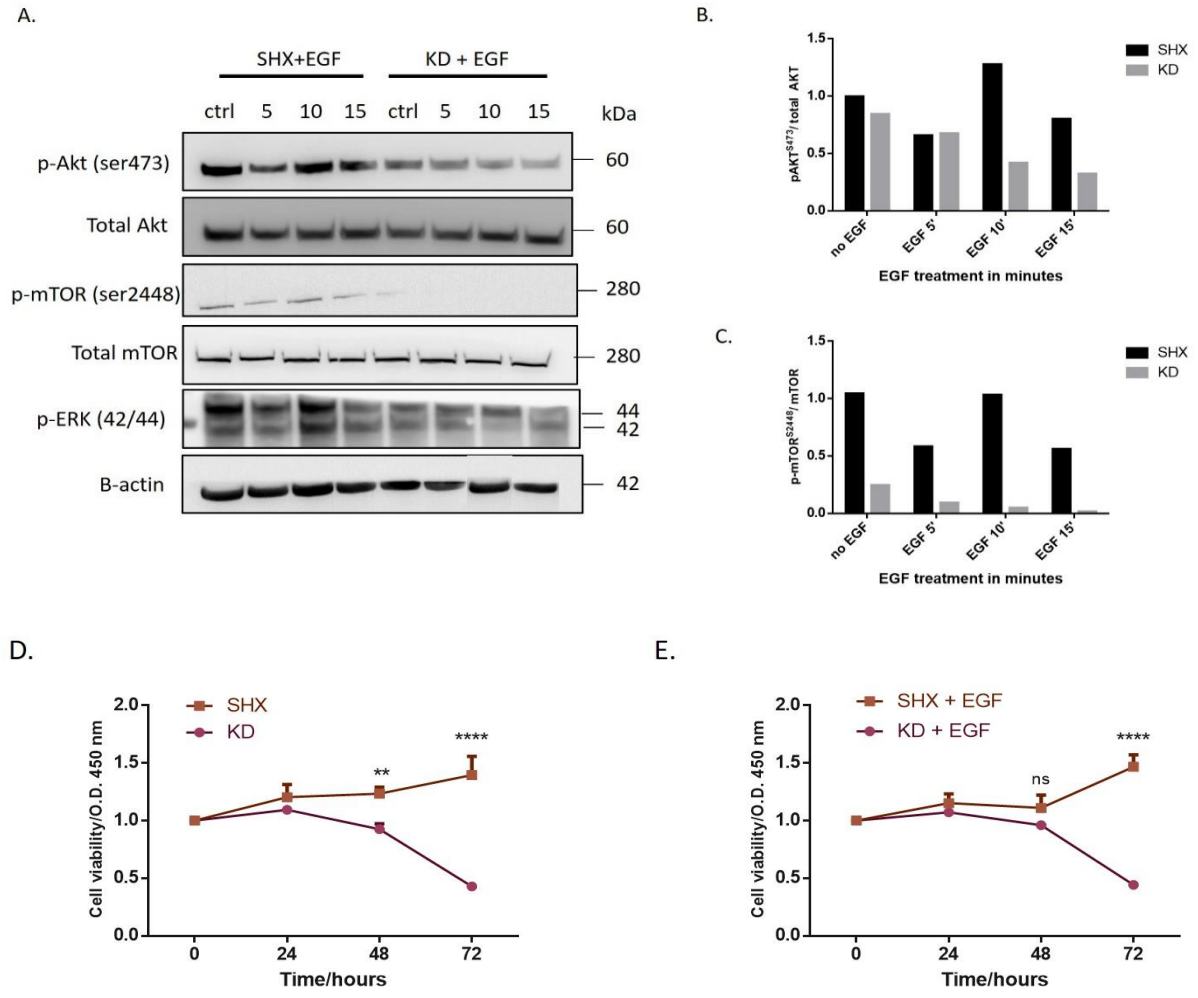


Figure 19*. Impact of PLCβ1 silencing on EGF mediated oncogenic response. A. Both PLCB1 KD and empty vector SHX U87MG cells were treated with 5ng/µl of EGF at various time points up to 15 minutes. Western blot analysis shows that p-Akt, p-mTOR and p-ERK expression were decreased in PLCβ1 knockdown cells as compared to empty vector cells. B. Graphical analysis sowing quantification of the expression of p-Akt/total Akt and C. p-mTOR/total mTOR. Densitometric analyses were conducted using image J. D. Viability assay was conducted on U87MG cells serum starved overnight and E. Serum starved and treated with 5ng/µl of EGF at various time points. Viability assay was proceeded at t0, t24, t48, and 72hrs. Data is representative of mean and sem. **** p=0.0001 and ** p=0.0025, 2-way ANOVA following Sidak's multiple comparison test with 95% confidence level was used in determining statistical differences.*

8. **Plasma membrane PtdIns(4,5)P_2 is affected by PLCβ1 expression in glioblastoma cells**

Due to relationship between PtdIns(4,5)P_2 and PLCβ1, as its substrate for hydrolysis into DAG and $InsP_3$, as well as the implication of PtdIns(4,5)P_2 in cell migration, an immunofluorescence staining was performed to determine the effect of PLCβ1 knockdown on the levels of PtdIns(4,5)P_2 at the plasma membrane. U87MG GBM cells were grown to 70% confluency in chronic serum complete media and fixed with 4% paraformaldehyde before staining. PLCβ1 knockdown cells expressed high levels of plasma membrane PtdIns(4,5)P_2 as compared with empty vector control cells.

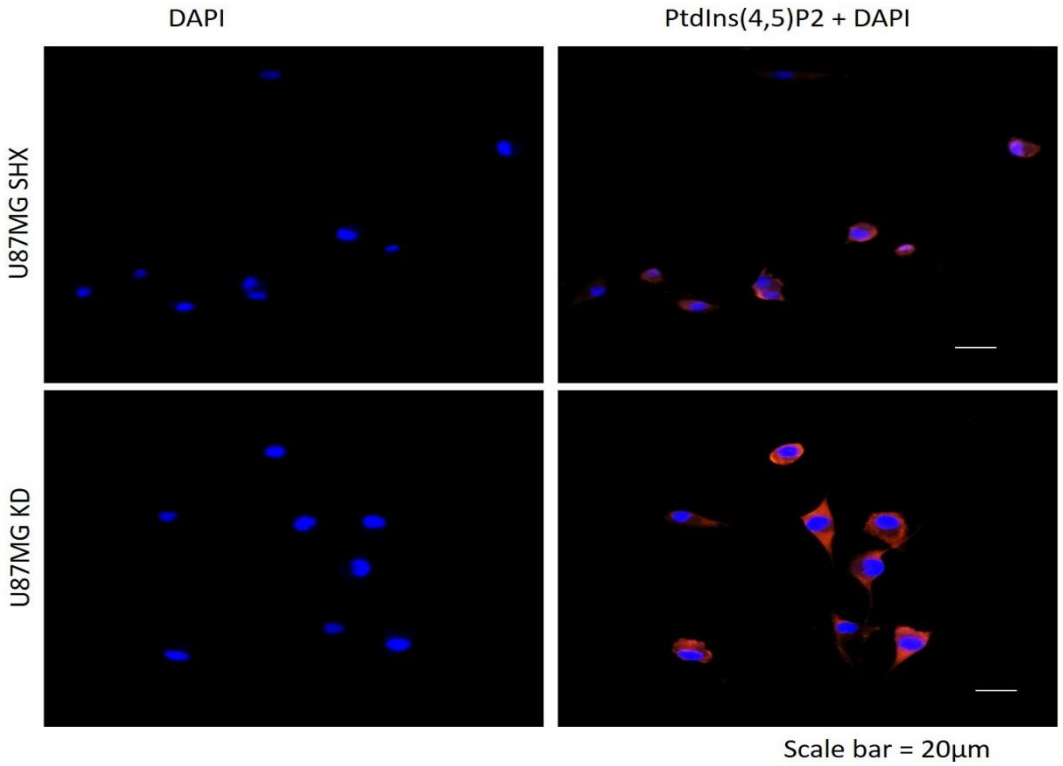


Figure 20. *Impact of PLCβ1 expression on plasma membrane PtdIns(4,5)P_2 levels. Immunostaining of plasma membrane PtdIns(4,5)P_2 shows that PLCβ1 knockdown promotes the increment of PtdIns(4,5)P_2 levels at the plasma membrane as compared to empty vector U87 GBM cells. Dapi stained the nucleus in blue and PtdIns(4,5)P_2 is stained at the plasma membrane in red.*

9. The effect of reducing SKIP expression on nuclear PtdIns(4,5)P_2

SC-35 and/or nuclear PtdIns(4,5)P_2 were measured by immunostaining in U-251 MG cells genetically manipulated to reduce expression the of SKIP.

In U251shSKIP cells, nuclear PtdIns(4,5)P_2 immunostaining was significantly higher as compared to that of shScramble cells (Figure 21A). Similarly, an increase in SC-35 was observed in shSKIP cells as compared to the shScramble cells. Using Image J to evaluate the corrected total cell fluorescence (CTCF), the correlation coefficient between nuclear PtdIns(4,5)P_2 and SC-35 were calculated; r = 0.184 in U251shScramble cells and r = 0.5694 in shSKIP cells (Figure 21B). Thus suggesting that the two nuclear signals PtdIns(4,5)P_2 and SC-35 do not constantly follow each other in all cells.

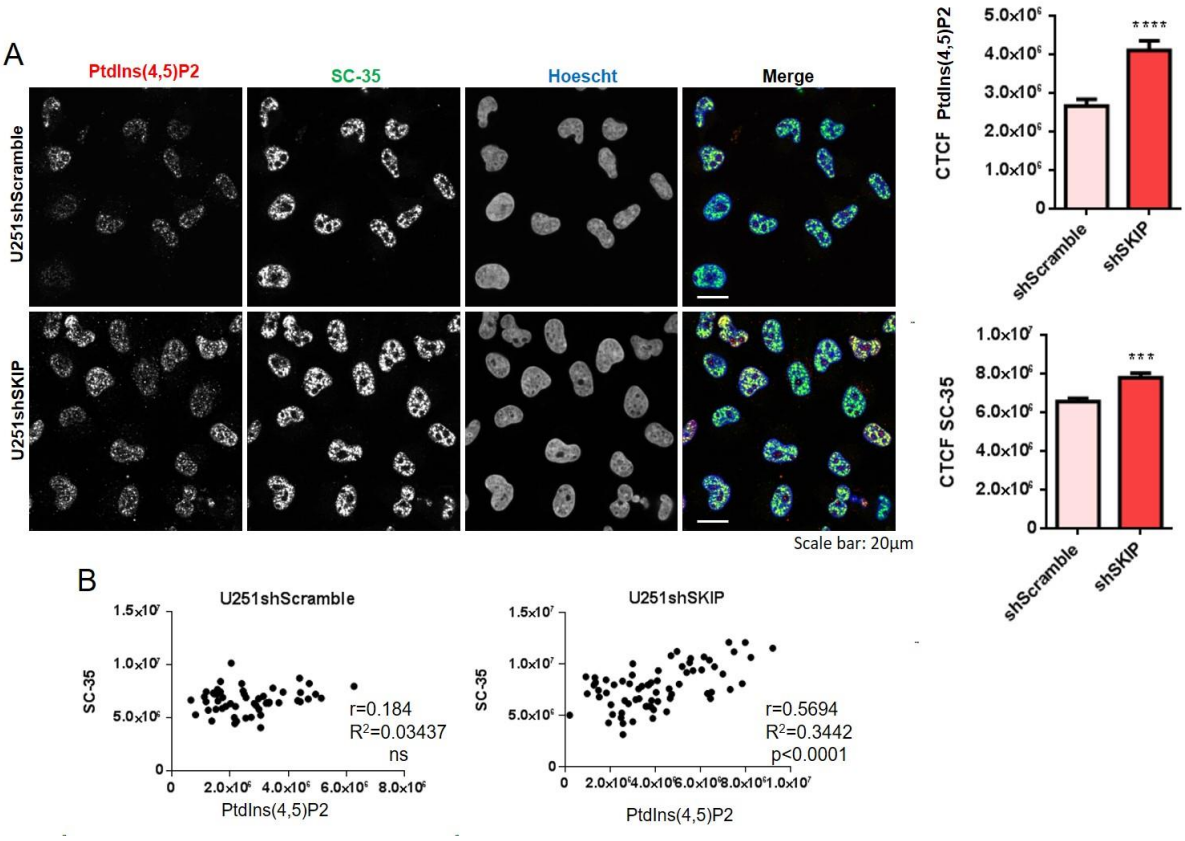


Figure 21. *Effects of SKIP on PtdIns(4,5)P_2 and SC-35 in the nuclear speckles of GBM U-251 MG cells. A. Nuclear PtdIns(4,5)P_2 was stained in red (Streptavidin NL557 conjugated), SC-35 in green (Alexa Fluor 488) and nuclei in blue (Hoechst 33342). The nuclear signals of both PtdIns(4,5)P_2 and SC-35 were quantified at 63x as CTCF. Error bars represent mean ± SEM (>25 cells analyzed per condition). The data shown are representative of at least three independent experiments. *** p = 0.0003; ****p < 0.0001 (Non-parametric and unpaired student's t-test). Scale bars 20 µm. Images were captured with confocal microscope, Zeiss LSM780, 63x/1.46 NA oil objective. B. Graphical representation of the correlation between PtdIns(4,5)P_2 and SC-35 staining in the nuclei.*

In addition, a siRNA transfection method was employed to silence SKIP in GB138 GBM cells (Fig 22). Using two different siRNAs for SKIP, it was determined that nuclear PtdIns(4,5)P_2 levels were reduced as compared to control siRNA. Moreover, one of the siRNAs, siRNA_1, also reduced SC-35 in GB138 cells as compared to siRNA control.

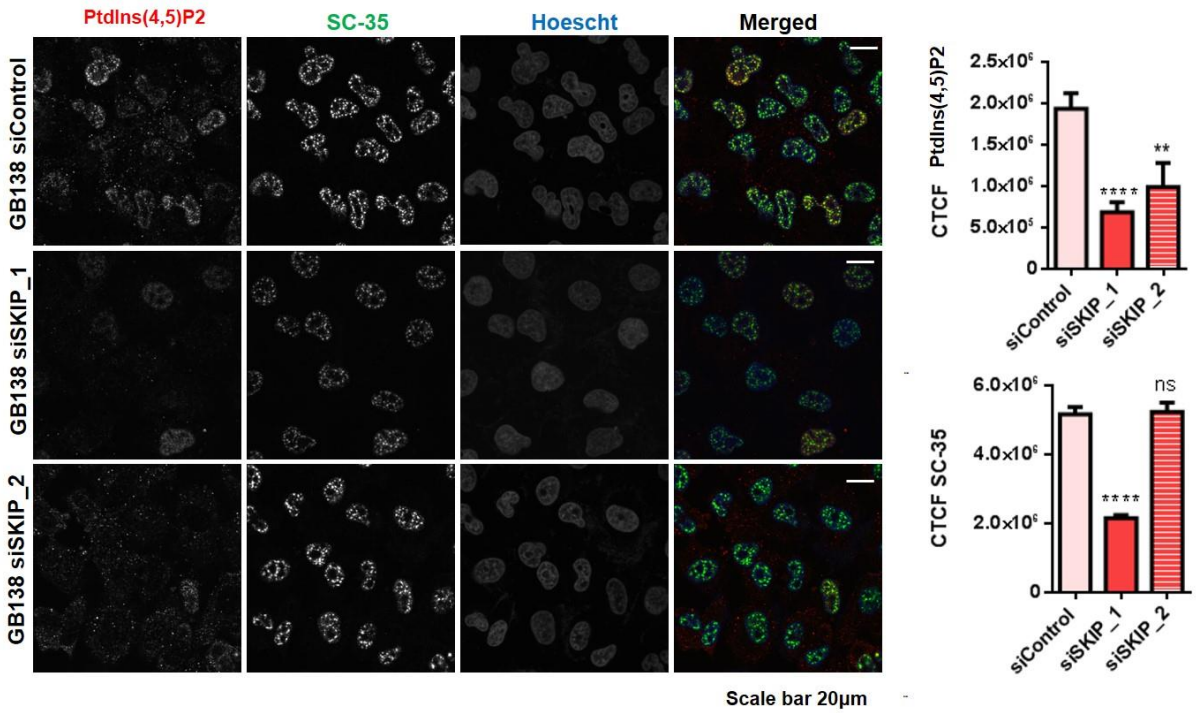


Figure 22. *Impact of SKIP on PtdIns(4,5)P_2 and SC-35 levels in the nuclear speckles of GBM GB138 cells. Nuclear PtdIns(4,5)P_2 was stained in red (Streptavidin NL557 conjugated), SC-35 in green (Alexa Fluor 488) and nuclei in blue (Hoechst 33342). The PtdIns(4,5)P_2 and SC-35 signal were quantified at 63x as CTCF. Error bars represent mean ± SEM (>30 cells were analyzed per condition). The data are representative of at least three independent experiments. ****p < 0.0001 (Non-parametric and unpaired student's t-test). Images were captured with confocal microscope, Zeiss LSM780, 63x/1.46 NA oil objective.*

10. Localization of SKIP in the nuclear speckles of GBM cells

SKIP indeed localizes within the nucleus, partly in speckles, as it co-localizes with SC-35 in U-251 MG GBM cells (Figure 23). Based on the analysis of at least 23 different cells in exactly three independent experiments, about 48% of nuclear SKIP staining co-localize with SC-35. This data also confirms the reduction of nuclear SKIP staining in shSKIP cells when compared to control cells (Figure 23).

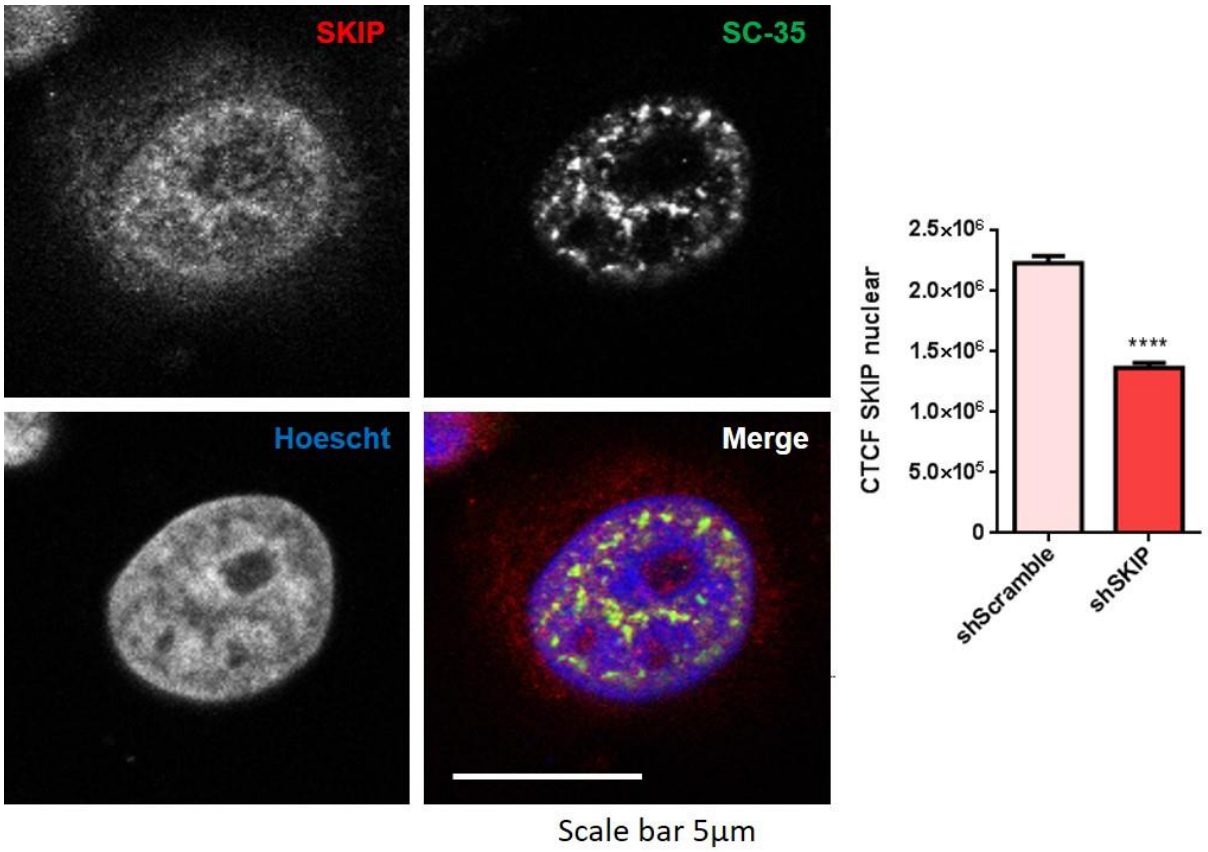


Figure 23. *Localization of SKIP to the nuclear speckles GBM U-251 MG cells. Cells kept in chronic serum for 24h were fixed with 4% paraformaldehyde before immunostaining. SKIP was stained in red (Streptavidin NL557 conjugated), SC-35 in green (Alexa Fluor 488) and nuclei in blue (Hoechst 33342). To evaluate the level of expression of SKIP in speckles, SC-35 was used as a mask. Based on the analysis of at least 23 cells in two independent experiments, 48% of nuclear SKIP co-localized with SC-35, hence in the nuclear speckles. The SC-35 and SKIP signals were quantified at 63x as CTCF. Error bars represent mean ± SEM ***p = 0.0005 (Non-parametric and unpaired student's t-test). Images were captured with confocal microscope, Zeiss LSM780, 63x/1.46 NA oil objective.*

11. Anti-tumoral role of SKIP in U87MG xenograft SCID mice

Using U87MG xenograft SCID mice as an *in vivo* model, U87MGshSKIP cells generated significantly larger tumors compared to control shScramble cells (Figure 24 A-C). In addition, a cell viability study revealed that shSKIP cells were more viable as compared to shScramble cells (Figure 24D). Using a BrdU incorporation-based proliferation assay, there was no significant difference in proliferation between ShScramble and shSKIP U87MG cells (Figure 24E). Finally, using FACS analysis of activated caspase 3, apoptosis was decreased in shSKIP cells as compared to U87MGshScramble cells (Figure 24F).

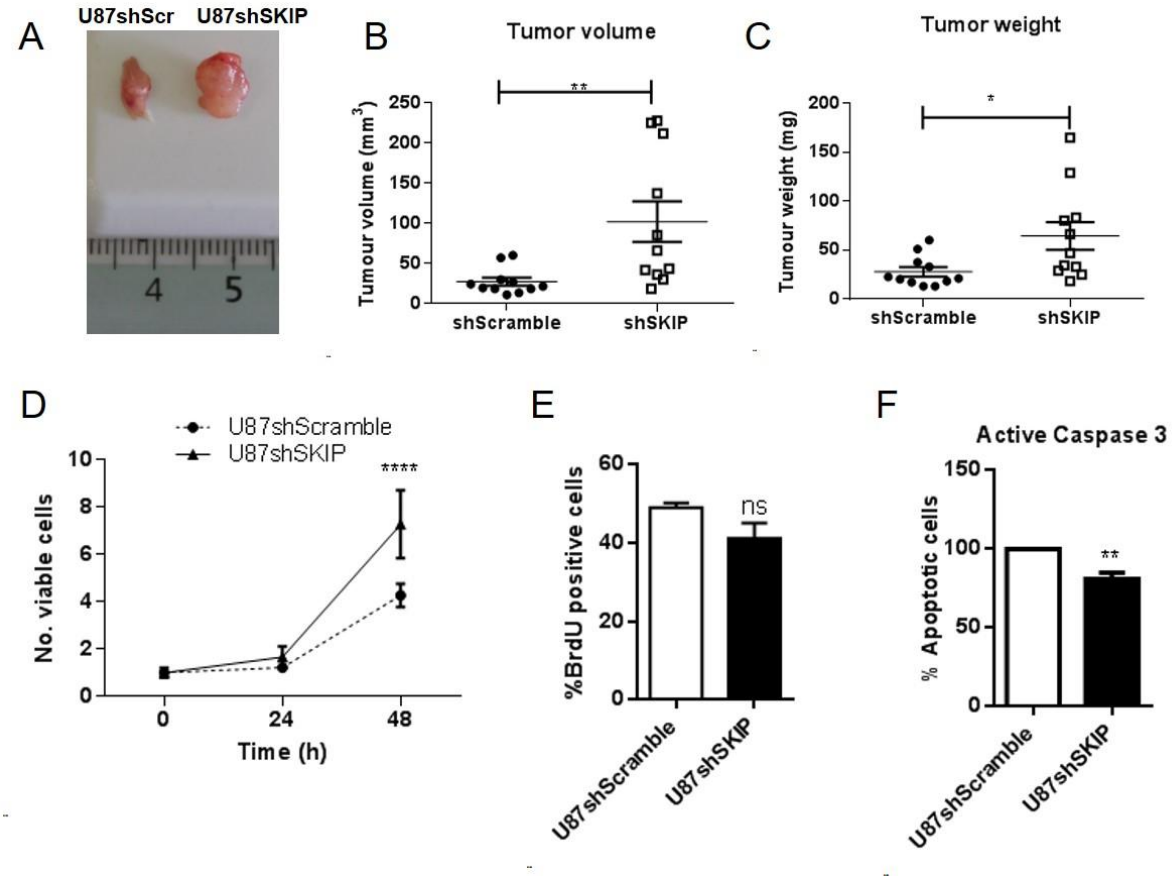


Figure 24. *The effects of SKIP reduction on tumor growth in vivo in U87MG xenograft model of GBM. A. Tumors generated after 5 weeks of xenografting in three different SCID mice shows a significant increase in tumor volume of shSKIP model B. and tumor weight (C) as well as in tumors generated by injecting shSKIP cells as compared to shScramble. (D) Graphical representation of the number of viable cells generated from a WST1 cell viability assay performed with U87MGshScramble and U87MGshSKIP cells. The data are expressed as mean ± SEM and standardized to time 0 h. Statistical analysis was performed with two-way ANOVA by followed by Sidak's multiple comparison post-hoc test, ****p < 0.0001. (E) Graphical*

*representation of BrdU incorporation-based proliferation in shScramble and shSKIP. The data are expressed as a mean percentage ± SEM, student's t-test followed by Mann Whitney test was used to determine the statistical significance. (F) Graphical representation of a FACS analysis of active caspase 3 showing the percentage of apoptotic cells in shSKIP compared to shScramble cells. The data are mean ± SEM which represents three independent experiments, **p = 0.0424.*

DISCUSSION

Despite the extensive studies on GBM worldwide, current GBM treatment therapies involving surgery, chemotherapy and radiotherapy are still not successful in curing the disease. As such, GBM patients usually experience very poor prognosis with only about 3-5% surviving up to 3 years or more (Louis et al., 2016; Lu et al., 2016). A large-scale gene profile studies on GBM revealed that several genes involved in cell cycle, cellular proliferation, migration, invasiveness, and survival are altered in GBM (Lu et al., 2016), explaining why existing therapies are not effective. The survival of GBM patients depends on understanding the molecular and key signaling events associated with these altered physiological processes in GBM.

Phosphoinositide metabolic enzymes, especially phospholipases and phosphatases, have been reported to participate in several cancer associated mechanisms, spanning from cell proliferation, differentiation, migration, apoptosis, to RNA synthesis (Owusu Obeng et al., 2020; Ramos et al., 2019b). PLCβ1, which is one of the most studied phospholipases, expressing at both nuclear and plasma membrane levels, has been documented to alter the expression of essential molecules like PtdIns(4,5)P_2, DAG, PKC, and calcium which mediates several oncogenic pathways and functions. A previous *in silico* study reported that PLCβ1 is a candidate signature gene for the proneural subtype of GBM and that PLCβ1 expression is inversely correlated with glioma pathological grades (Lu et al., 2016).

Based on this previous analysis, this study firstly aimed at confirming this by using a different *in silico* analysis platform, GEPIA to confirm the expression levels of PLCβ1 in GBM, low grade glioma, and normal control samples. Indeed, PLCβ1 levels were significantly reduced in GBM samples as compared to control samples. Furthermore, PLCβ1 expression was higher in low grade glioma samples compared to GBM samples, hence also confirming that PLCβ1 expression levels are inversely correlated with glioma pathological grades; it reduces as pathological grades increase. More importantly, this study delved deeper to confirm the findings from *in silico* analysis through the use of clinical GBM, low grade astrocytoma, and oligodendroglioma while comparing with control samples. First and foremost, mRNA expression of PLCβ1 in 20 clinical GBM samples confirmed that PLCβ1a and 1b are decreased. On the other hand, other PLCs such as PLCγ1, and PLCδ3 were significantly higher than in

control samples. In fact, PKCα, which is activated downstream of PLCβ1, also increased in GBM samples. This may be attributed to a compensation in the loss of PLCβ1, where other PLCs like PLCγ1 and PLCδ3 increase. Notably, these other PLCs can activate PKCα downstream (Allen et al., 1997; Lattanzio et al., 2019).

Furthermore, for the first time, using clinical samples from low grade and GBM patients, this study confirms that PLCβ1 expression in glioma is indeed inversely correlated to pathological grades, as PLCβ1 mRNA expression was higher in low grade oligodendroglioma and astrocytoma samples compared to GBM samples. Consistently, a recent study on 30 glioma clinical samples revealed a higher expression of PLCβ1 in the glioma samples compared to control samples. Moreover, the authors concluded that miR-423-5p inhibits proliferation and metastasis of glioblastoma cells by targeting directly the 3'-UTR of PLCβ1 gene (Zhao et al., 2019), suggesting that PLCβ1 may regulate essential cancer-related cellular mechanisms in GBM.

Following this, PLCβ1 was knocked down in U87MG GBM cells. Migration was increased in PLCβ1 knockdown cells, and immunofluorescence staining showed differences in F-actin expression in these cells with respect to control cells. PLCβ1 knockdown cells indeed possessed enlarged and stretched F-actin compared to more roundish F-actin in control cells. In addition, PLCβ1 knockdown cells possessed well established and prominent filopodia while none was observed in control cells. Filopodia are actin-based membrane protrusions formed at the leading edge of migrating cells to support the migration of these cells. Migrating cells undergo a series of tightly regulated adhesion dynamics to the ECM and neighbouring cells. Increase in cell adhesion corresponds to increase cell spreading through the formation of lamellopodia, which ultimately results in increased cell migration. Usually, in the absence of lamellipodia, which is also actin-based membrane structures that protrude and retract to promote migration, possession of only filopodia can support a residual, slow migration in cancer cells (Krause and Gautreau, 2014). Consistent with this data, a transwell assay revealed that cell migration is potentiated in PLCβ1 knockdown cells as compared to control cells. Numerous deaths associated with cancer are primarily related to the migratory or metastatic potential of tumors to invade new regions different from original tumor sites. Single or tumor cell clusters separate from primary tumors, assume migratory abilities, and invade new regions via their surrounding matrices. Moreover, some PLC isoforms have been reported to

participate in this process in several cancer types (Bertagnolo et al., 2007; Chen et al., 1996; Sengelaub et al., 2016).

PtdIns(4,5)P_2 is an established PLCβ1 substrate which is known to control actin dynamics and cell migration (Audhya and Emr, 2003; Fiume et al., 2019). In fact, this study showed that plasma membrane PtdIns(4,5)P_2 levels were increased in PLCβ1 knockdown cells. In 1321N1 GBM cells, an increase in PtdIns(4,5)P_2 levels promoted cell migration as SHIP2 was genetically silenced (Ramos et al., 2018) and this is consistent with other findings where silencing of PLCβ1 increases plasma membrane PtdIns(4,5)P_2 levels. Hence, PtdIns(4,5)P_2 plays an essential role in cancer cell migration.

On the other hand, cell to ECM adhesion in PLCβ1 knockdown cells was significantly higher than control cells. It is well known that during cancer metastasis, cells detach from original tumor site, remodel surrounding ECM via alterations in ECM dynamics, and degradation of surrounding ECM to allow their invasion of new sites. Lamellipodia and or filopodia based migration has been shown to constantly rely on cell-ECM dynamics during migration. This involves a well-regulated process of disassembly and recycling of old cell–ECM adhesion points, while forming new adhesion sites at the leading edge, this controlled balance is essential for effective cell migration (Conway and Jacquemet, 2019). This was consistent with the activation of focal adhesion kinase (FAK) on tyrosine 397, which is an established focal adhesion marker. Importantly, the mean length or size of focal adhesions has been shown to predict cell speed and migration (Kim and Wirtz, 2013). Already, previous studies have shown that a depletion or reduction in FAK activation decreases focal adhesion turnover and subsequently cell motility (Ilić et al., 1995).

Interestingly, the ability of epithelial cells to assume mesenchymal phenotypes may also explain why migration is higher in PLCβ1 knockdown cells. Epithelial cells interacting with the basal membrane via their basal surfaces undergo various biochemical transformations leading to achievement of the mesenchymal cell phenotype, including elevated production of extracellular matrix, enhanced motility, invasiveness and increased resistance to apoptosis (Iser et al., 2017; Iwadate, 2016). Epithelial cells remain in direct contact with the underlying basement membrane and adhere to other neighbouring cells and ECM by aid of cell junctions; they also express epithelial markers. When undergoing EMT, epithelial cells become polarised and subsequently change to motile and ECM component-secreting mesenchymal cells. In the

intermediate transition stages, cells may express both epithelial and mesenchymal markers, this stage is regarded as partial EMT. Once transition is complete, the cells would have already shed all epithelial markers and only express mesenchymal markers (Iser et al., 2017; Iwadate, 2016). Slug and Twist are essential transcriptional factors known to regulate mesenchymal phenotypes, including the expression of mesenchymal markers such as vimentin, as well as the involvement in ECM deposition. Hence, PLCβ1 downregulation in GBM drives EMT in GBM cells by promoting the expression of Slug and Twist. Downstream, vimentin was confirmed to be highly expressed in PLCβ1 knockdown cells with respect to control samples.

So far, everything points toward the fact that PLCβ1 downregulation in GBM promotes aggressive phenotypes, characterised by increased cell migration, EMT and patients' survival. However, when GBM cells were treated with EGF, Akt and mTOR activations were decreased in PLCβ1 knockdown cells. GBM is characterised by EGFR amplifications and protein overexpression, even several inhibitors for EGFR are under consideration to be used in GBM therapy (Westphal et al., 2017). EGF activation, which is downstream of EGFR signaling, has been shown to promote the activation of downstream essential oncogenic pathways such as the PI3K/Akt and RAS/MAPK signaling pathways in an EGFR dependent manner to facilitate cell migration, cell proliferation and cell survival in GBM (Chen et al., 2017). Therefore, it is interesting to observe that PLCβ1 downregulation reduces EGF-induced Akt and mTOR activation, which are pro-survival pathways, and viability with or without EGF. Hence, it is possible that PLCβ1 downregulation in GBM may have a dual impact in GBM by promoting GBM cell migration and EMT but also reducing EGF induced GBM cell survival.

As for the impact of SKIP in GBM, just as PLCβ1, lowering SKIP in GBM cells increased PtdIns(4,5)P_2, but in nuclear speckles in this case. Nuclear speckles, also termed as inter chromatin granule clusters are made up of RNA and proteins that usually serve as structural domains capable of regulating RNA transcription, splicing and mRNA export (Galganski et al., 2017). Aside from the plasma membrane expression of PtdIns(4,5)P_2 as seen in PLCβ1, it has also been reported to be expressed in the speckles of several cells where it co-localizes with the serine/arginine splicing factor SC-35, normally used as a nuclear speckle marker (Galganski et al., 2017). Nuclear PIs are critical signaling nodes implicated in several nuclear processes such as RNA processing involving RNA export or pre-mRNA processing and DNA transcription. PIs localize to the nucleus with their metabolic enzymes, thus creating a PI-cycle

independent of the plasma membrane (Cocco et al., 1987; Divecha et al., 1991; Payrastre et al., 1992). This study confirmed that the nuclear SC-35 expression followed the same trend as SKIP in U-251 MG cells, as lowering SKIP induced a reduction in SC-35. In contrast, lowering SKIP in GB138 GBM cells rather lowered nuclear PtdIns(4,5)P_2 and SC-35. Even though the mechanism by which nuclear PtdIns(4,5)P_2 is altered by SKIP is not yet understood, it is possible that SKIP phosphatase activity on nuclear PtdIns(4,5)P_2 may be cell type-dependent. In addition, there is a chance that nuclear SKIP may use nuclear pools of PtdIns(4,5)P_2 as a substrate in some cells. Nuclear SKIP may also interact or form complexes with some nuclear proteins that regulate the activities of Type I or Type II PI kinases or most essentially PLC isoforms present in nuclear speckles in order to effect changes in nuclear PtdIns(4,5)P_2 (Ramos et al., 2020). Importantly, SKIP staining revealed that 48% of SKIP colocalize with SC-35 in the nuclear speckles, thus explaining why SKIP had an impact in SC-35 staining. However, it is certainly clear why the alteration of SC-35 in SKIP lowered cells differ in U-251 MG and GB138 cells. A previous study reported that SC-35 staining varies among different cells (Galganski et al., 2017).

SHIP2, just as SKIP, is another PI-5 phosphatase which has been shown to play pro-tumoral roles in other cancer types. In xenograft mice, MDA MB 231 cells with SHIP2 deletion produces significantly smaller tumors and less metastasis observed in lung sections compared to controls (Ghosh et al., 2018). As such, this study investigated whether SKIP would also be pro-tumoral in xenograft mice. In xenograft mice, U87MG shSKIP cells produced significantly larger tumors in xenograft mice compared to controls. In addition, shSKIP cells were more viable but more apoptotic than control cells. Interestingly, SKIP did not affect cell proliferation as evidenced by BrdU assay. Therefore, SKIP produced opposite effects in GBM as opposed to SHIP2 in the breast cancer model (Ghosh et al., 2018). This study is limited in a way that it does not explain or investigate how SKIP is able to alter tumor growth and viability. However, the ability of SKIP to alter PtdIns(4,5)P_2 may explain why changes in SKIP expression affects apoptosis. A study demonstrated that some pools of PtdIns(4,5)P_2 regulates apoptosis by directly influence the activation of initiator caspases 8 and 9, and their downstream effector caspase 3 (Mejillano et al., 2001). Intriguingly, it has also been established that SKIP phosphatase activity does not affect PtdIns(3,4,5)P_3 levels in U87MG and U-251MG GBM cells as the reaction product, PtdIns(3,4)P_2 was not changed during SKIP alteration (Ramos et al.,

2019a). As to the pro-tumoral role of SKIP in GBM, it has also been shown that SKIP reduction in U-251 MG cells and LN 229 GBM cells induced a decrease in cell migration velocity. On top of this, PtdIns(4,5)P_2 levels was upregulated in SKIP depleted GBM cells upon their adhesion on fibronectin. In fact, SKIP and PtdIns(4,5)P_2 co-localized in ruffles of these cells upon fibronectin adhesion. Hence, it may be possible that SKIP regulates fibronectin-dependent GBM cell migration and PtdIns(4,5)P_2 may be involved (Ramos et al., 2019a).

Conclusion

So far, this study has shown several different ways by which PLCβ1 can influence cell migration, that is through actin reorganization and lamellipodia formation, PtdIns(4,5)P_2 expression, focal adhesion and cell-ECM adhesion, and EMT. Indeed, this confirms that cancer cell migration is a complex topic to understand. On the other hand, this study has shown that PLCβ1 may have dual impact in GBM as evidenced in EGF-induced experiments. Hence, the ability of PLCβ1 to influence all these essential cellular processes underscore the need to increase research on PLCβ1 signaling in GBM. Important things to note for future studies include whether there are some compensating mechanisms promoting the upregulation of other PLCs during the knockdown or downregulation of PLCβ1. The use of PI3K inhibitors on PLCβ1 knockdown cells to confirm whether the increase in cell migration is not attributed to PI3K. It would also be interesting to use only PTEN negative cells with PLCβ1 downregulation to confirm this theory. In EMT, further studies can investigate whether epithelial markers like E-cadherin, ZO-1, and Occludins are indeed lost in PLCβ1 cells while the cells attain increment of mesenchymal markers like α smooth muscle actin, fibronectin, and N-cadherin. Moreover, investigating the ability of PLCβ1 to self-renew and attain other mesenchymal features may be beneficial. In EGF-induced oncogenic cellular processes, the impact of PLCβ1 on cell migration, apoptosis, cell cycle, and proliferation can also be studied to provide clarity on PLCβ1-EGF activities. Essentially, this research will benefit significantly from the development of PLCβ1 isoform specific inhibitors, which may be helpful to confirm all the data observed in genetically engineered cells.

SKIP phosphatase activity alters the nuclear levels of PtdIns(4,5)P_2 and SC-35. The association between SKIP and other nuclear proteins such as MAD2L1BP may account for SKIP's nuclear localization, affecting speckle formation because of the alteration in SC-35, and other fundamental nuclear functions. Since both SKIP and PLCβ1 downregulation induces an increase in PtdIns(4,5)P_2, meaning both uses PtdIns(4,5)P_2 as a substrate, are there any possible interactions between the two? Moreover, it would be interesting to investigate whether SKIP downregulation does not trigger compensatory functions by other PI-5 phosphatases like SHIP2. Based on this study, PLCβ1 and SKIP are essentially non-redundant proteins, especially in GBM pathogenesis.

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