Insights into the Molecular Mechanisms of Phospholipase D-Mediated Cancer Cell Survival

By

Ronald Chase Bruntz

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Approved:

John H. Exton, M.D., Ph.D.

H. Alex Brown, Ph.D.

Heidi E. Hamm, Ph.D

Kevin C. Ess, M.D., Ph.D.

Daniel C. Liebler, Ph.D.

Brian E. Wadzinski, Ph.D.

For my parents Sam and Judy, my sister Melissa,

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LIST OF ABBREVIATIONS

2-DG	2-Deoxyglucose
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
AIG	Anchorage independent growth
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ANT	ADP/ATP nucleotide translocase
ATG	Autophagy related gene
ATP	Adenosine triphosphate
cAMP	cyclic AMP
CL	Cardiolipin
Co-IP	Co-immunoprecpitation
CR	Conserved region
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle's medium
DN	Dominant negative
DOCK2	Dedicator of cytokinesis 2
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase

FBS	Fetal bovine serum
FRB	FKBP12-rapamycin Binding domain
GAP	GTPase activating protein
GBM	Glioblastoma multiforme
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
IgG	Immunoglobulin G
IP	Immunoprecipitation
IP_3	Inositol trisphosphate
LC3	Microtubule-associated protein 1A/1B-light chain 3
LPA	Lysophosphatidic acid
LRPPRC	Leucine-rich pentatricopeptide repeat-containing protein
MAPK	Mitogen activated protein kinase
MPB	Maltose binding protein
MMP	Matrix Metalloproteinase
mTOR	Mammalian target of rapamycin
myrAkt1	myristoylated Akt1
NF1	Neurofibromin 1
NRTK	Non-receptor tyrosine kinase
p21Cip1	p21 cyclin dependent kinase inhibitor
p70S6K1	Ribosomal p70 S6K kinase
PAK	p21 activated protein kinase

PAS	Preautophagosomal structure
PC	PhosphatidIcholine
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PE	Phosphatidylethanolamine
PEA15	Phosphoprotein enriched in astrocytes of 15 kDa
PH	Pleckstrin homology
PI	Phosphoinositide
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol trisphosphate
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKN	Protein kinase N
PKC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PP1	Protein phosphatase 1
PtdBuOH	Phosphatidylbutanol
PtdOH	Phosphatidic acid
PTEN	Phosphatase and tensin homologue
PX	Phox homology
RTK	Receptor tyrosine kinase
S1P	Spingosine 1-phosphate
SDS	Sodium dodecyl sulfate

SH2	Src homology 2
SH3	Src homology 3
SOS	Son of sevenless
SEM	Standard error of the mean
TAP	Tandem affinity purification
TCA	Trichloroacetic acid
tf-LC3	Tandem-fluorescent LC3
WASp	Wiskott–Aldrich Syndrome Protein

CHAPTER I

INTRODUCTION

History and classification

Phospholipase D (PLD) activity was first described in plant studies when Hanahan and Chaikoff reported the metabolism of nitrogen containing phospholipids into phosphatidic acid (PtdOH) and choline in carrots (enzyme commission number EC3.1.4.4) (Hanahan and Chaikoff, 1947a). Further investigation using carrot (Hanahan and Chaikoff, 1947b) and cabbage (Hanahan and Chaikoff, 1948) extracts confirmed the presence of an enzyme capable of hydrolyzing phosphatidylcholine (PC) when added exogenously to the plant preparation. These early studies paved the way for the purification of a soluble PLD from cottonseed extracts (Tookey and Balls, 1956). In addition to hydrolyzing phospholipids such as PC, PLD enzymes catalyze a transphosphatidylation reaction in the presence of primary alcohols where the phosphatidyl group from PC is transferred to the alcohol instead of water to generate a phosphatidyl alcohol at the expense of PtdOH (Yang et al., 1967) (Figure 1). Thus, the gold-standard technique for measuring PLD activity *in vivo* is to measure production of metabolically stable phosphatidyl alcohols in the presence of primary alcohols such as butanol or ethanol (Brown et al., 2007). Almost thirty years after the initial description of PLD in plants, Saito and Kanfer provided the first evidence of a PLD activity in a mammalian tissue by partially purifying a PLD enzyme from rat brain particulate fractions (Saito and Kanfer, 1975; 1973). PLD enzymes are now known to be ubiquitously expressed and PLD activity has been described in almost all organisms from viruses and prokaryotes up to fungi, plants, and higher eukaryotes such as humans (reviewed in Selvy et al., 2011).

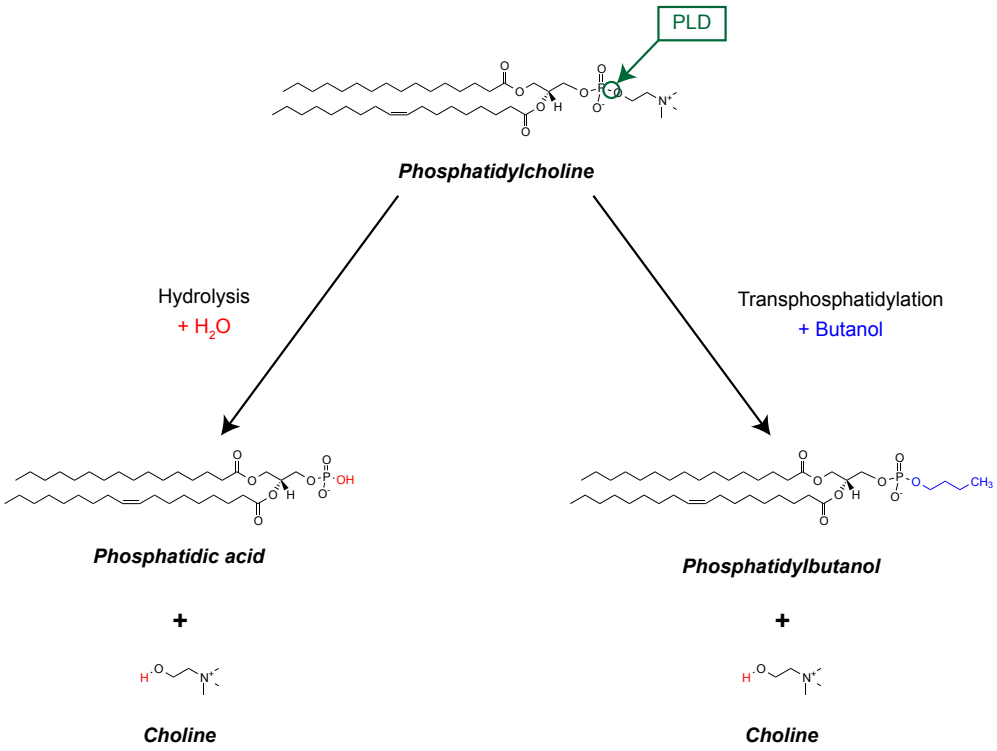

Figure 1. PLD hydrolysis and transphosphatidylation. Shown are substrates and products of PLD-catalyzed hydrolysis in the presence of water, or transphosphatidylation in the presence of butanol.

PLD was first cloned from castor beans (Wang et al., 1994), and the sequence information enabled other groups to clone PLD enzymes from many other organisms. To date, over 4000 sequences for PLDs from various organisms have been deposited into the NCBI GenBank (Selvy et al., 2011). Two PLD isoforms have been cloned in humans and are commonly referenced as PLD1 (Hammond et al., 1995) and PLD2 (Lopez et al., 1998). PLD1 and PLD2 orthologues have also been cloned from mice (Colley et al., 1997a; 1997b) and rat (Kodaki and Yamashita, 1997; Park et al., 1997). Although many PLD enzymes, both prokaryotic and eukaryotic, were initially described based on their ability to hydrolyze PC, cloning and subsequent sequence analyses revealed the truly diverse nature of these enzymes. The overall sequence homology between plant, yeast, and mammalian enzymes is quite low, with only four small regions of sequence similarity

termed conserved regions (CR) I, II, III, and IV (Morris et al., 1996). CR II and CR IV contain phospholipase catalytic sequences, termed HKD domains (Koonin, 1996), characterized by the sequence $HxKx_4Dx_6G(G/S)$ where x's denote amino acids between the histidine, lysine, and aspartic acid residues. Based on sequence analyses of PLDs from various organisms, enzymes with the characteristic HKD catalytic domain are categorized as part of a PLD "superfamily" and include PLD enzymes from prokaryotes, fungi, plants, and mammals (Koonin, 1996; Ponting and Kerr, 1996). Additionally, non-PLD enzymes with HKD domains such as endonucleases (Pohlman et al., 1993), cardiolipin synthase (CLS) (Ivanisevic et al., 1995), and phosphatidylserine synthase (PSS) (DeChavigny et al., 1991) are included in the PLD superfamily. Not all HKD enzymes share all four conserved regions as PLD from *Streptomyces* contains only CR I, II, and IV, with I and IV being the most similar to eukaryotic PLD and the CLS/PSS bacterial enzymes contain only regions I and IV (Morris et al., 1996). As will be discussed below, these HKD enzymes are all believed to share a similar reaction mechanism.

In addition to the PC-hydrolyzing PLDs, several other PLDs have been identified and cloned in humans. Glycosylphosphatidylinositol PLD (GPI-PLD), a non-HKD PLD, hydrolyses GPIs to produce an inositol glycan and PtdOH and functions primarily to release GPI-anchored proteins from membranes (Schofield and Rademacher, 2000). N-acyl phosphatidylethanolamine PLD (NAPE-PLD) is another non-HKD PLD that hydrolyzes NAPE to produce PtdOH and N-acylethanolamine, which is further metabolized into anandamide, a ligand for cannabinoid receptors (Okamoto et al., 2004). Autotaxin, or lysophospholipase D, is a non-HKD PLD that hydrolyzes lysophospholipids, such as lysophospholipase C (LPC) to produce the potent mitogen, lysophosphatidic acid (LPA) (Houben and Moolenaar, 2011). In addition to these non-HKD PLD enzymes, a mitochondrial PLD, mitoPLD or PLD6, was recently cloned and

shown to encode one copy of the HKD catalytic sequence (Choi et al., 2006). MitoPLD is believed regulate mitochondrial fusion by hydrolyzing mitochondrial cardiolipin (CL) instead of PC to produce PtdOH (Choi et al., 2006). Although these non-HKD/non-canonical PLD mediate important biological events, the remainder of this dissertation will focus on the mammalian PLD enzymes.

Structure and mechanism

Human PLD1 has three known splice variants termed PLD1a, PLD1b (Hammond et al., 1997), and PLD1c (Steed et al., 1998). PLD1b is 38 amino acids shorter than PLD1a and appears to have similar regulatory and catalytic properties (Hammond et al., 1997). By contrast, PLD1c contains an early truncation mutation and has been theorized to function as an inhibitor of endogenous PLD activity (Steed et al., 1998). Likewise, splice variants for PLD2 have been published, but little is known about their functions *in vivo* (Steed et al., 1998). Full-length PLD1 and PLD2 share approximately 50% sequence identity and have similar domain structures. As such, PLD1 and PLD2 seem to exhibit the same substrate preferences, namely mono- and di-unsaturated PC (Pettitt et al., 2001). At their amino termini, PLD1/2 contain tandem phox homology (PX) and pleckstrin homology (PH) domains (Steed et al., 1998; Sung et al., 1999b), which are known to mediate interactions with lipid membranes (Lemmon, 2008) and are believed to regulate PLD localization within the cell (Figure 2). The PX/PH domains were defined by predicted secondary structures, as there is little primary sequence similarity to other known PX/PH proteins. PLD from lower eukaryotes such as yeast (Rose et al., 1995) and plant (Qin and Wang, 2002) also encode PX and PH domains. These domains are not required for catalytic activity as PX/PH truncation mutants are catalytically active *in vitro* (Sung et al., 1999a). Conserved regions I-IV, including the two catalytic HKD domains, are C-terminal to the PX/PH domains and vary in length among the different

PLD isoforms. The PLD1 isoforms contain a unique "loop" region between CRII and CRIII of approximately 120 amino acids in PLD1a; this loop is 38 amino acids shorter in PLD1b (Hammond et al., 1997). This function of this loop region is unknown, but it is believed to inhibit the enzyme *in vivo* since deletion of this region results in a more highly active protein *in vitro* (Sung et al., 1999b). The function of the carboxy terminus of the protein is also unknown, but any mutations to this end of the enzyme results in a catalytically inactive protein, suggesting that this region participates in formation or stabilization of the active site (Liu et al., 2001).

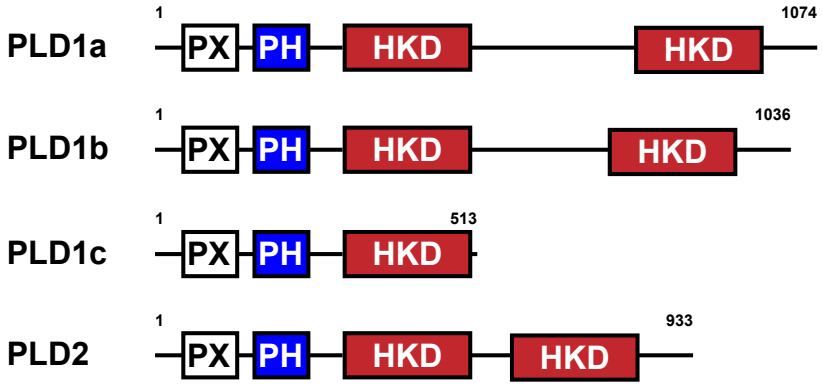

Figure 2. Schematic of human PLD isoforms and splice variants. Human PLDs encode amino-terminal PX and PH domains followed by two catalytic "HKD" domains, characteristic of the PLD superfamily. PLD1a and PLD1b vary by 38 amino acids in a "loop" region between the two HKD domains. The PLD1c splice variant contains an early truncation mutation resulting in an inactive protein. Numbers indicate amino acid positions.

Early studies using strategically radiolabeled substrates and intermediates suggested that PLD enzymes use a two-step "ping pong" reaction mechanism where a covalent phospho-enzyme intermediate is created before release of the product (Jiang et al., 1984; Stanacev and Stuhne-Sekalec, 1970). This reaction mechanism was supported by structural studies using *Yersinia pestis* murine toxin (Rudolph et al., 1999) and nuc endonuclease (Gottlin et al., 1998), both members of the HKD-containing PLD

superfamily. In the first step, a histidine residue from one HKD domain serves as a nucleophile to attack the phosphate group of substrate PC to form a phosphatidyl histidine. The histidine from the second HKD domain donates protons to the choline leaving group to assist in formation of the phosphatidyl histidine. The second histidine then abstracts protons from water and the activated water molecule hydrolyzes the phosphatidyl histidine intermediate to release PtdOH (Rudolph et al., 1999). In this reaction scheme, alcohol is a better nucleophile than water and so transphosphatidylation is preferred reaction over hydrolysis in the presence of alcohol. Crystal structures of the HKD-containing PLD from *Streptomyces* (PMF-PLD) and the HKD-containing nuc endonuclease (Stuckey and Dixon, 1999) have been solved and support these previously established biochemical insights into the molecular mechanisms of PLD hydrolase activity. Likewise, crystal structures for PMF-PLD demonstrated the two HKD domains situated adjacent to each other along an axis of symmetry. Although nuc contains only one HKD domain, it crystallized as a homodimer with the two HKD domains similarly situated along an axis of symmetry with the HKD domains coming together to form the active site. The crystal structures thus supported the mechanism of catalytic histidine residues. The exact functions of the lysines and aspartic acids are unknown, but they are believed to stabilize the phosphatidyl histidine intermediate as has been reported with similar enzymes such as nucleoside diphosphate kinase (Morera et al., 1995).

Localization and tissue distribution

The PLD enzymes are ubiquitously expressed and are found in almost all mammalian tissues. mRNA analyses demonstrated that PLD1 is highly enriched in human heart, brain, pancreas, uterus, and intestine and PLD2 is highly enriched in brain, placenta, lung, thymus, prostate, and uterine tissue (Lopez et al., 1998). Early studies

into PLD subcellular localization were limited to measurements of PLD enzymatic activity following subcellular fractionation (Edwards and Murray, 1995). Molecular cloning allowed for characterization of PLD1 and PLD2 subcellular localization through the use of fluorescent fusion proteins and isoform-specific antibodies to track endogenous protein. Many studies examining PLD subcellular localization have overexpressed PLD1 or PLD2 and this has led to some controversy over localization of endogenous proteins. However, the work of multiple, independent investigators has produced a relatively consistent pattern of PLD1 and PLD2 localization.

Under resting conditions, PLD1 resides on perinuclear, intracellular membranes of secretory vesicles, lysosomes, endosomes, Golgi, and endoplasmic reticulum (Brown et al., 1998a; Du et al., 2003; Freyberg et al., 2001)Freyberg 2001, Du 2003, (Colley et al., 1997b; Du et al., 2004). On the other hand, PLD1 is basally localized to the plasma membrane in neuroendocrine cells such as chromaffin (Vitale et al., 2001) and PC12 cells (Du et al., 2003), although PC12 cells express very low levels of endogenous PLD1 (Gibbs and Meier, 2000). IgE stimulation of RBL-2H3 mast cells (Brown 1998) or PMA stimulation of fibroblasts (Kim et al., 1999b) and COS-7 cells (Du et al., 2003) results in PLD1 relocalization to the plasma membrane suggesting that PLD1 activation might require relocalization following extracellular stimulation. As such, mutations that prevent post-translational palmitoylation and plasma membrane recruitment reduce PMA stimulated PLD1 activity (see “Regulation of PLD”). The varied and dynamic subcellular localization patterns of PLD1 correlate with a diverse functional roles.

In contrast to PLD1, PLD2 primarily localizes to plasma membranes under basal conditions. Studies measuring overexpressed (Colley et al., 1997b; Du et al., 2004; O'Luanaigh et al., 2002) and endogenous PLD2 (Du et al., 2004; Park et al., 2000; Sarri et al., 2003) support a plasma membrane localization pattern. PLD2 is enriched in detergent-insoluble, lipid-raft fractions (Czarny et al., 1999; Zheng and Bollinger Bollag,

2003), which contain clusters of cell-surface receptors and other signal transduction molecules (Simons and Toomre, 2000). Agonist stimulation typically results in PLD2-receptor co-localization both at the plasma membrane and in endocytic vesicles and PLD2 has been proposed to participate in receptor endocyosis and recycling (Du et al., 2004). Additionally, stimulation of fibroblasts with serum (Colley et al., 1997b) or mast cells with antigen (O'Luanaigh et al., 2002) results in PLD2 relocalization to filopodia and membrane ruffles, respectively. Thus, the subcellular localization patterns of PLD2 suggest functional roles ranging from signal transduction to cytoskeletal reorganization (see "Functions of PLD").

Regulation of PLD

Regulation by lipids

Phosphoinositides (PI)

The gold standard *in vitro* PLD activity assay measures the release of a tritiated choline headgroup from an isotopically-labeled PC substrate, typically as part of a vesicle containing PC, phosphatidylethanolamine (PE), and phosphatidylinositol 4,5-bisphosphate (PIP_2). The development of this assay required optimization of lipid vesicle substrate compositions and PIP_2 was absolutely required to detect choline hydrolysis from PLD purified from HL-60 membranes (Brown et al., 1993). Recombinant expression of mammalian PLD1 (Hammond et al., 1997; Min et al., 1998) and PLD2 (Colley et al., 1997b; Kodaki and Yamashita, 1997; Lopez et al., 1998) revealed that both enzymes require PIP_2 for their activity *in vitro*. Besides PIP_2, other phosphatidylinositol species can stimulate PLD activity. For example, $PI(4,5)P_2$ and $PI(3,4)P_2$ robustly stimulate human PLD1b activity with phosphatidylinositol 3,4,5-trisphosphate (PIP_3) able to stimulate activity to about half that of the PIP_2 species (Hammond et al., 1997; Hodgkin

et al., 2000). Similarly, PLD1b displays approximately 2-fold higher binding affinity for PIP_2 than PIP_3. Phosphatidylinositol phosphate (PIP) and $PI(3,5)P_2$ are much less effective stimulators of PLD activity and suggests that the position of the phosphates on the inositol ring is critical for maximal activity (Hodgkin et al., 2000). For reasons not immediately clear, rat PLD1 is stimulated equally by $PI(4,5)P_2$ and PIP_3 but not by $PI(3,4)P_2$ (Min et al., 1998), suggesting that different species might display some selectivity for PIP_n species *in vivo*.

PIP_2 is believed to interact with two distinct sites on PLD; one in the PH domain and one between the catalytic domains and each site is believed differentially regulate enzyme activity. Multiple groups have shown that the PX and PH domains of PLD1 (Henage et al., 2006; Sung et al., 1999b) and PLD2 (Sung et al., 1999a) are not required for enzyme activity. Since PIP_2 is required for PLD activity, investigators reasoned that there must be another PIP_2 binding site outside of the PH domain. Mutational analysis narrowed PIP_2 binding to a polybasic region between CRII and CRIII and when arginine 554 and 558 of PLD2 (corresponding residues are R691 and R695 for human PLD1a) were mutated, both the *in vitro* catalytic activity and PIP_2 binding were severely compromised (Sciorra et al., 1999). Plant PLDs also require PIP_2 and a similar binding region was established for plant-PLD1β. Mutation of lysines K437 and K440, also between CRII and CRIII ablated PLDβ activity *in vitro* (Zheng et al., 2002). PIP_2 binding induces a conformational shift in the catalytic domain of plant PLDβ that helps recruit PC to the active site (Zheng et al., 2002), suggesting that PIP_2 binding to the region between catalytic domains might influence catalytic activity by promoting PC substrate binding. Later studies demonstrated that in the absence of PIP_2, PLD was unable to bind PC vesicles, further supporting the role of PIP_2 in PC binding.

Although mutation of the PIP_2 binding region between CRII and CRIII significantly decreases PLD catalytic activity, these mutations do not impact PLD localization as

measured by immunofluorescence or subcellular fractionation (Sciorra et al., 1999). PH domains are known to bind phosphatidylinositols and regulate subcellular localization (Lemmon, 2008). As such, PLD-PH domain binding to PIP_2 is a means for regulating subcellular localization. Aligning the PH domain from PLD2 with PH domains from other proteins revealed several conserved residues believed to mediate PIP_n binding. When arginine 236 and tryptophan 237 of PLD2 (corresponding residues are R317 and W318 in PLD1a) were mutated, the resulting protein was catalytically inactive *in vivo* but displayed similar catalytic activity to wild-type protein when PLD2 was immunoprecipitated and assayed *in vitro* (Sciorra et al., 2002). PLD2 resides primarily in detergent-insoluble membrane fractions but mutation of R237 and W238 resulted in relocalization of PLD2 to detergent-soluble membrane fractions. Mutation of PLD2 also resulted in a relocalization from plasma membranes to intracellular localizations (Sciorra et al., 2002). Parallel studies with PLD1b also demonstrated mislocalization following deletion of the PH domain (Hodgkin et al., 2000). The role of PIP_2 *in vivo* might be to recruit PLD to a specific membrane and to enhance catalysis by promoting substrate binding to the active site. However, the absolute requirement of PIP_2 for PLD activity was established *in vitro* and some debate exists as to the exact nature of PIP_2 regulating PLD *in vivo.*

Several lines of evidence support a role for PIP_2 in regulating PLD *in vivo*. The rate-limiting step of PIP_2 production is the phosphorylation of PI(4)P by PI(4)P 5-kinase (PIP5K) (Ling et al., 1989). When PIP5K was overexpressed in cells to increase levels of PIP_2, PLD activity also increased, suggesting higher PIP_2 levels increase PLD activity in intact cells (Divecha et al., 2000). Modulation of PIP_2 levels in the opposite direction also influences PLD activity. The antibiotic neomycin has a high affinity for PIP_2 and is used for cell signaling studies due to its ability to sequester PIP_2. As expected, treatment of cells with neomycin decreases PLD activity in a manner that is reversed when PIP_2 is

replenished in the system (Liscovitch et al., 1994). Similarly, synaptojanin, an inositol polyphosphate 5-phosphatase capable of dephosphorylating PIP_2 (McPherson et al., 1996), was purified as a cytosolic factor capable of inhibiting PLD activity *in vitro* (Chung, 1997). Although the original characterization of PLD inhibition by synaptojanin was performed *in vitro*, synaptojanin might function as a mechanism for terminating PLD signaling *in vivo*. Similarly, the actin and PIP_2 binding protein gelsolin inhibits PLD *in vitro* by binding and sequestering PIP_2 (Banno et al., 1999). Overexpression of gelsolin also inhibits PLD transphosphatidylation activity, further supporting a role for PIP_2 regulating PLD activity *in vivo* (Banno et al., 1999).

These studies create a model where PIs, specifically PIP_2, regulate PLD by influencing catalysis via an interaction in the PLD catalytic domains and by influencing membrane localization via an interaction in the PH domain. Furthermore, PX domains are also well-established PI binding domains (Song et al., 2001). The PLD PX domain appears to bind PIP_3 quite selectively over mono- and di-phosphorylated PIs and mutation of PLD1 arginine 179 substantially reduces PIP_3 binding (Stahelin et al., 2004). Several studies have shown a dependence on PIP_3 generation for PLD activation following sell surface receptor stimulation. Phosphoinositide 3-kinases (PI3K) produce PIP_3 by phosphorylating the D3 position of the inositol ring (Cantley 2002) and both PLD and PI3K activities are frequently stimulated following receptor tyrosine kinase stimulation (see “Cell-surface receptor regulation of PLD”). PI3K inhibitors decrease PLD activation following insulin receptor stimulation (Standaert et al., 1996) and mutation of the PIP_3 binding site on PLD (PLD1-R179) prevents PLD activation and membrane recruitment following platelet derived growth factor stimulation (PDGF) (Lee et al., 2005). Therefore, membrane recruitment of PLD is precisely regulated by both PIP_2 and PIP_3 through interactions with the PH and PX domains, respectively.

Fatty acids

Purification of various mammalian and plant PLD enzymes revealed activities that were stimulated differently by unsaturated fatty acids. Before cloning of the PLD enzymes, a PLD was purified from pig lung that was stimulated by unsaturated fatty acids such as oleic (18:1), lineoleic (18:2), and arachidonic (20:4) acids (Okamura and Yamashita, 1994). Several lines of evidence suggest that PLD2 is the isoform stimulated by unsaturated fatty acids. PLD activity is highly stimulated by oleate in Jurkat T cells but not HL-60 cells (Kasai et al., 1998). mRNA analysis suggests that Jurkat T cells express only PLD2 whereas HL-60 cells express PLD1. Likewise, PLD2 is highly enriched in the lung (Lopez et al., 1998) where this enzyme was initially purified. Finally, oleate stimulates PLD activity in RBL-2H3 mast cells when PLD2, but not PLD1 is overexpressed (Sarri et al., 2003). The *in vivo* relevance of unsaturated fatty acid stimulation of PLD2 is not fully understood.

Lipid modifications

Both PLD1 and PLD2 undergo lipid modification. By feeding tritiated fatty acids to cells and then measuring lipid incorporation onto PLD protein, investigators found that PLD1 is covalently modified with palmitic acid (Manifava et al., 1999). Later studies concluded that cysteine 240 and 241 are the amino acids responsible for attachment (Sugars et al., 1999). The exact function of these lipid modifications is not known, but the data to date suggest that proper subcellular localization, and not catalytic activity, requires these palmitoylation events. In COS-7 cells, PLD1 is normally localized to punctate intracellular membranes. However, when the palmitoylated cysteines were mutated to alanine, the levels of punctate intracellular PLD1 decreased with a concomitant increase in plasma membrane localized protein (Sugars et al., 1999). The mutant protein was less active *in vivo* but showed no differences in activity when

compared to wild-type protein *in vitro*, suggesting that the palmitoylation promotes accessibility of substrate lipids to PLD in the cell (Sugars et al., 1999). As discussed below, epidermal growth factor (EGF) stimulates PLD activity in a variety of cell lines. The cysteine to alanine mutants are much less responsive to EGF stimulation than wild-type protein, suggesting that palmitoylation is required for cell surface receptor activation of PLD1 (Han et al., 2002b). PLD2 is also palmitoylated on C223 and C224 (Xie et al., 2002a). Similarly to PLD1, mutation of the cysteine residues decreases *in vivo* activity and also results in a smaller fraction of membrane-associated PLD2 (Xie et al., 2002a). Therefore, palmitoylation is one of the mechanisms by which PLD is properly localized under basal and stimulated conditions.

Phosphorylation

Serine and theonine phosphorylation

Many groups have detected phosphorylation of PLD1 and PLD2 at tyrosine, serine, and threonine residues, yet the functional significance of these events is not always clear and most likely depends on the cell system and stimulus under investigation. For example, protein kinase C (PKC) stimulates PLD1 activity through a direct protein-protein interaction and not via phosphorylation in fibroblast membranes (Conricode et al., 1992). By contrast, PKCα stimulation of PLD in neutrophil membranes requires adenosine triphosphate (ATP) and is inhibited by treatment with staurosporine, a non-selective protein kinase inhibitor (Lopez et al., 1995), suggesting that PKCα phosphorylates PLD directly or phosphorylates an intermediate protein that activates PLD. A proteomic analysis revealed that PMA-stimulated PKC phosphorylates S2, T147, and S561 on PLD1 (Kim et al., 1999a). When these residues were mutated, the authors measured a slight decrease in PMA-stimulated PLD activity *in vivo* but no changes in *in vitro* activity suggesting that phosphorylation of these residues is not required for

catalytic activity (Kim et al., 1999a). Although not required for activity, one study has suggested that phosphorylation of S2 is required for association with the actin cytoskeleton following cell-surface receptor activation (Farquhar et al., 2007). Similar proteomic analyses for PLD2 revealed that phorbol 12-myristate 13-acetate (PMA), a PKC activator, stimulation of COS-7 cells increased phosphorylation of S134, S146, S243, T72, T99, T100, and T252 and that S243 and T252 were the predominant sites of phosphorylation (Chen and Exton, 2005). Mutation of S243 and T252 completely inhibited binding of phospho-serine and phospho-threonine antibodies to PMA-stimulated PLD2, but did not inhibit PMA-stimulated PLD activity (Chen and Exton, 2005). PMA treatment of COS-7 cells results in rapid increases in both PLD1 (Hu and Exton, 2003) and PLD2 (Chen and Exton, 2004) activity. PLD1 (Hu and Exton, 2003) and PLD2 (Chen and Exton, 2004) phosphorylation increases only after much longer PMA exposure and this correlates to a decrease in PLD activity. The functional consequence of PKC phosphorylation of PLD1 and PLD2 is probably to downregulate PLD activity in these cells. This hypothesis is substantiated by observations that stausporine treatment prolongs PMA-stimulated PLD activity (Hu and Exton, 2003) and PKCα/β inhibitors block PLD phosphorylation but not PMA-stimulated PLD activity (Chen and Exton, 2004).

Other lines of evidence suggest that phosphorylation of PLD by PKC might be required for cell-surface receptor stimulation of PLD. EGF stimulation of COS-7 cells results in a rapid increase in both PLD1 T147 phosphorylation and activity that can be ablated by expression of dominant-negative (DN) PKCα or by mutating the PKC phosphorylation sites of PLD1 (Han et al., 2002b). The discrepancies between the functional consequences of PMA- and EGF-stimulated PLD phosphorylation might be explained by differences in PKC isoforms being activated under each condition. For example, PMA activates many PKC isoforms and EGF might only activate PKC isoforms

that positively regulate PLD activity, such as PKCα. In support of this hypothesis, PKCδ is believed to negatively regulate PLD1 activity in COS-7 cells (Han et al., 2002b). By contrast, PKCδ is believed to mediate the PMA-activation of PLD2 in PC12 cells (Han et al., 2002a) and integrin-stimulated PLD2 activation in COS-7 cells requires phosphorylation of S566 by PKCδ (Chae et al., 2010). Therefore, the functional role of PKC phosphorylation of PLD1 and PLD2 largely depends on the cell background, stimulus, and PKC isoform under investigation.

In addition to PKC, other serine/threonine kinases are known to regulate downstream PLD functions by phosphorylation-dependent mechanisms. For example, the p90 ribosomal S6 kinase phosphorylates PLD1 at T147 and this phosphorylation event is required for K^+ stimulated PLD activity and exocytosis in PC12 neuroendocrine cells (Zeniou-Meyer et al., 2008). Additionally, AMP-activated protein kinase (AMPK) phosphorylates PLD1 at S505 (Kim et al., 2010). Glucose withdrawal stimulates AMPK and PLD activity and the phosphorylation of S505 by AMPK is required for glucose-stimulated PLD activity *in vivo*. Mutation of S505 has no effects on basal PLD activity suggesting that the phosphorylation is required for catalytic activity (Kim et al., 2010). Likewise, PLD2 regulates insulin secretion following EGF stimulation in rat insulinoma cells via a mechanism that requires cyclin dependent kinase 5 (Cdk5) phosphorylation of PLD2 S134 (Lee et al., 2008). Mutation of S134 to alanine partially decreases EGF stimulated PLD2 activity, but does not inhibit basal PLD2 activity (Lee et al., 2008). Casein Kinase II (CKII) is found in complex with both PLD1 (Ganley et al., 2001) and PLD2 (Ahn et al, 2006) and phosphorylates both isozymes. The exact function of the CKII phosphorylation events is unknown, but PLD catalytic activity is unaffected. Together, these studies suggest that phosphorylation of PLD most likely functions to alter PLD subcellular localization and availability to substrate since basal activity *in vivo* and PLD activity *in vitro* is not sensitive to mutation of phosphorylation sites.

Tyrosine phosphorylation

Tyrosine phosphorylation as a mechanism of PLD regulation was first proposed after the observation that vanadate, a tyrosine phosphatase inhibitor, increased PLD activity in HL-60 granulocytes (Bourgoin and Grinstein, 1992). In later studies, PLD was shown to be tyrosine phosphorylated as measured by immunoprecipitation (IP) of PLD and immunoblotting with a phospho-tyrosine antibody (Gomez-Cambronero, 1995). Stimulation of the G-protein coupled receptor (GPCR) for the chemotactic peptide fMLP in neutrophils increased PLD activity and tyrosine phosphorylation in a manner that was inhibited by general tyrosine kinase inhibitors, suggesting that tyrosine phosphorylation directly regulates PLD activity (Gomez-Cambronero, 1995). These studies, however, did not discriminate between direct activation of PLD by tyrosine phosphorylation and activation of PLD by upstream regulatory proteins. Later studies indicated that tyrosine phosphorylation of PLD2 influences catalytic activity to some extent since dephosphorylation of immunoprecipitated PLD2 with purified tyrosine phosphatases can decrease (Henkels et al., 2009) or increase (Horn et al., 2005) catalytic activity *in vitro*, suggesting that the two phosphatases used in these studies are dephosphorylating distinct tyrosine residues.

EGF stimulation results in tyrosine phosphorylation of PLD2 (Min et al., 2001) at Y11 (Slaaby et al., 1998) and Y296 (Henkels et al., 2010), presumably through EGF receptor-mediated phosphorylation of PLD2, although the possibility of an intermediate kinase was not completely eliminated. When Y11 was mutated to alanine, the authors measured a 2-fold increase in EGF-stimulated PLD activity, suggesting that phosphorylation of Y11 functions to down-regulate the PLD2 response (Slaaby et al., 1998). Likewise, mutation of Y296 to phenylalanine also increased *in vivo* PLD2 activity (Henkels et al., 2010), suggesting that like Y11, Y296 also functions to downregulate PLD2 activity. Other tyrosine kinases such as JAK3 and Src have been reported to

phosphorylate PLD2 at Y415 and Y511, respectively (Henkels et al., 2010). In this study, purified JAK3 or Src was incubated with cell lysate and PLD2 was immunoprecipitated to determine which tyrosine sites were phosphorylated. The authors did not address the possibility that JAK3 or Src might be activating an intermediate kinase that could phosphorylate PLD2 and so caution must be exercised when interpreting these results. Regardless, the authors suggested that phosphorylation of Y415 stimulates PLD activity whereas phosphorylation of Y511 is inhibitory (Henkels et al., 2010). These studies suggest that the level of PLD2 activity between various cell lines might result from different ratios of tyrosine phosphorylation on both activating and inhibitory residues.

Besides modulating catalytic activity, tyrosine phosphorylation is known to regulate PLD2-protein interactions. As discussed below, PLD2 couples EGF stimulation to activation of the small G-protein Ras by binding the adaptor protein Grb2 and activating son of sevenless (Sos), a guanine nucleotide exchange factor (GEF) for Ras. Grb2 interacts with PLD2 at Y169 and mutation of nearby tyrosine Y179 to phenylalanine increases the interaction of Grb2 with Y169 and enhances activation of Ras signaling pathways (Di Fulvio et al., 2006). The interaction of Grb2 with Y169 increases PLD2 catalytic activity and mutation of Y169 to phenylalanine results in a catalytically inactive protein (Di Fulvio et al., 2006). Furthermore, Akt kinase was suggested to phosphorylate threonine 175 of PLD2 (Di Fulvio et al., 2008). The authors used a relatively non-specific Akt-kinase substrate antibody to probe IP'ed PLD2 instead of using purified, recombinant Akt kinase. Regardless, mutation of T175 to alanine inhibited the Y179F mutant from stimulating Ras, suggesting that T175 and Y179 function to fine-tune Ras signaling through PLD2 (Di Fulvio et al., 2008).

As tyrosine kinases are frequently upregulated in cancer, discovering precise molecular targets of tyrosine kinases further enhance understanding of cancer biology. A recent proteomic study examined changes in global phospho-tyrosine residues in cells

overexpressing the constitutively active and transforming nucleophosmin-anaplastic lymphoma kinase (NPM-ALK). PLD1 and PLD2 had increased phosphorylation at Y711 and Y573, respectively, in cells overexpressing NPM-ALK (Wu et al., 2010). Future studies will be required to determine the functional significance of these and other novel phosphorylation events on PLD.

Protein activators of PLD

PKC

Phorbol esters such as PMA potently stimulate PLD activity in many cell lines and tissues (Exton, 1999). These compounds function as diacylglycerol (DAG) mimetics and potently stimulate the conventional (DAG and Ca^{2+} responsive; α, β_1, β_2, γ) and novel (DAG responsive; δ, ε, η, θ) PKC isoforms (Nishizuka, 1984), thus suggesting that PMA stimulates PLD through a PKC-dependent mechanism. In early studies linking PMA, PKC, and PLD, investigators separated fibroblast membranes, containing PLD, from cytosol, containing PKC, and showed that PMA did not stimulate membrane PLD activity unless purified PKC was included in the reaction (Conricode et al., 1992). In the same study, PKC was shown to stimulate PLD activity in a kinase-independent manner. Additionally, PKC stimulation of PLD was potentiated by PMA, leading to the hypothesis that activated PKC stimulates PLD through a protein-protein interaction, independent of kinase activity. Multiple groups confirmed a direct interaction using purified proteins (Lee et al., 1997b) and by co-immunoprecipitation (co-IP) of PLD and PKC from cell lysates (Siddiqi et al., 2000). Interestingly, the interaction is enhanced following PMA stimulation, suggesting that activated PKC is a better stimulator of PLD than non-activated PKC. The requirement for activated PKC suggests that cell surface receptors might stimulate PLD through a PKC-dependent mechanism and many reports have

indeed demonstrated a requirement for PKC in PLD activation following receptor stimulation (see “Cell-surface receptor regulation of PLD”).

The conventional PKC isoforms, namely α, β_1, and β_2, but not PKCδ, stimulate PLD from fibroblast and HL-60 membranes (Conricode et al., 1994; Ohguchi et al., 1996) and purified PLD from heterologous expression systems (Min et al., 1998). Purified conventional PKC stimulates PLD1a and PLD1b indistinguishably *in vitro* (Hammond et al., 1997) whereas PLD2 is unresponsive to PKC stimulation (Colley et al., 1997b). The interaction between PKC and PLD1 is complex as several lines of evidence suggest that at least two sites on PLD1 mediate the interaction with PKC. Expression of a PLD1 deletion mutant lacking the first 325 amino acids results in a construct that is catalytically active, but unresponsive to PMA stimulation, which suggests that PKC stimulates PLD activity through an interaction with the N-terminus (Sung et al., 1999b). However, the N-terminally truncated PLD1 still co-IPs with PKC, suggesting another site of interaction somewhere in the C-terminus (Sung et al., 1999b). The N-terminal binding site was narrowed to a region between amino acids 50-115 (Park et al., 1998; Zhang et al., 1999) and the C-terminal binding region on PLD1 was determined to be between amino acids 325-582 (Park et al., 1998).

The mechanism by which PKC stimulates PLD1 activity is also complex and requires interaction with both the N- and C-terminal interaction sites. A detailed kinetic analysis of the activation of PLD1 by PKC revealed that PKC is a mixed activator of PLD1. PKC increases substrate binding (decrease in K_m value) and also increases catalytic activity (increased k_{cat} value). An N-terminally truncated PLD1 lacking the first 311 amino acids was much less responsive to PKC stimulation than full-length PLD1 protein *in vitro*. The kinetic analysis suggested PKC still increased k_{cat} of the N-terminally truncated protein but had no effect on substrate binding (Henage et al., 2006). Thus, PKC modulates individual kinetic parameters through distinct interactions on PLD1. The

unique kinetic properties of PKC explain how other activators of PLD, such as the small GTPase Arf, synergize with PKC to robustly stimulate PLD1 activity (Singer et al., 1996).

Although PMA and PKC stimulate PLD1 activity *in vitro* through a direct protein-protein interaction, the regulation of PLD by PKC *in vivo* is more complex. As discussed above, PKC phosphorylates both PLD1 and PLD2 and these phosphorylation events are generally believed to inhibit PLD activity and may function to terminate PLD signaling after cell-surface receptor activation. Additionally, PMA stimulation can also increase PLD2 activity *in vivo* (Colley et al., 1997b; Han et al., 2002a; Siddiqi et al., 2000), although the fold-stimulation of PLD2 by PMA is much less than the fold-stimulation of PLD1. Given the wide range of PKC effectors, PMA-stimulation of PLD2 may be explained by other intermediate proteins instead of a direct protein-protein interaction. In certain cells such as PC12 cells, PLD2 stimulation by PMA appears to require the activity of PKCδ as a dominant-negative PKCδ construct inhibits PMA-induced PLD2 activation (Han et al., 2002a). Since purified PKCδ did not stimulate PLD1 (Conricode et al., 1994), more evidence is needed to determine whether PKCδ stimulates PLD2 directly or through an intermediate protein. In addition to PKCδ, PKCε was also shown to regulate PLD2 activation following sphingosine 1-phosphate (S1P) stimulation in lung epithelial cells (Gorshkova et al., 2008). A direct protein-protein interaction was not measured and an intermediate protein is likely mediating the effects of PKCε on PLD2.

Arf

The ADP-ribosylation factor (Arf) proteins are small molecular weight (M_r=21kDa) GTPases first identified as the factors needed for ADP ribosylation of the $G\alpha_s$ heterotrimeric G-protein by cholera toxin (Kahn and Gilman, 1984). Arfs have since been implicated in a variety of cellular events but are predominantly involved in vesicle

formation and trafficking along with the cytoskeletal and membrane rearrangements that accompany these events (Moss and Vaughan, 1998). Arf was originally identified as a cytosolic factor capable of stimulating PLD activity in HL-60 cell membranes. In those experiments, the addition of cytosol and a non-hydrolysable GTP analog (GTPγS) to HL-60 cell membranes robustly stimulated PLD activity. The observation that GTPγS was required for this stimulatory activity strongly implicated a GTPase as the activating factor. The factor was purified, sequenced, and identified as Arf1/3 (Brown et al., 1993; Cockcroft et al., 1994). There are 6 known Arf family members in humans, Arf 1-6, and are divided into three classes based on size, sequence, and gene structure; class I (Arf1-3), class II (Arf 4-5), and class III (Arf 6) (Moss and Vaughan, 1993). All six Arf proteins are capable of directly stimulating PLD activity, although with varying efficiencies (Massenburg et al., 1994). Arf proteins also stimulate PLD activity indirectly by stimulating PIP5K activity to increase local PIP_2 levels (Honda et al., 1999). The Arf-stimulated increase in PIP_2 and PtdOH levels through PIP5K and PLD, respectively, might function to terminate Arf signaling as the GAP activities of several ARF-GAPS are synergistically stimulated by PIP_2 and PtdOH (see “Function of phosphatidic acid”).

Prior to cloning PLD1 and PLD2, chromatographic separation of PLD activities from rat brain revealed two distinct PLD activities where one activity was stimulated by Arf and the other by oleate, most likely PLD1 and PLD2, respectively (Massenburg et al., 1994). *In vitro* studies using purified proteins later showed that Arf1 and Arf3 are capable of stimulating PLD1a and PLD1b between 10-40 fold compared to unstimulated PLD1 (Hammond et al., 1997; Lopez et al., 1998; Min et al., 1998). PLD2 is either completely unresponsive (Colley et al., 1997b) or modestly responsive (approximately 2-fold) (Lopez et al., 1998; Sung et al., 1999a), suggesting that Arfs 1 and 3 stimulate PLD1 specifically. However, as discussed in later sections, Arfs 4 and 6 have been implicated in regulating PLD2 activity *in vivo* (Caumont et al., 1998; Kim et al., 2003b). The sites of

Arf-interaction on PLD have not been determined conclusively, although the amino terminus of PLD1 is dispensable for Arf stimulation and kinetic analyses of an N-terminal deletion mutation revealed that Arf was a catalytic activator of PLD (Henage et al., 2006; Sung et al., 1999b). Interestingly, when the amino terminus of PLD2 is deleted, Arf-stimulates PLD activity nearly as much as PLD1 (Sung et al., 1999a). This suggests that sequestration of the PLD2 N-terminus, by lipid or protein binding or by post-translational modification, might free an Arf binding site that is otherwise sterically hindered on PLD2. As an example, the GM_2 ganglioside activating protein binds PLD1 and PLD2 and significantly enhances the responsiveness to Arf (Nakamura et al., 1998; Sarkar et al., 2001). Likewise, proteins such as Arfaptin, which bind and sequester Arf (Tsai et al., 1998), inhibit PLD activity and emphasize the importance of Arf for regulating PLD activity.

Although the Arf-interaction site has not been determined for PLD, several groups have screened Arf mutants that still bind GTP and downstream effectors but do not stimulate PLD activity. Deletion of the first 17 amino acids of Arf1 or mutation of asparagine 52 to arginine in the switch I region renders the protein unable to stimulate PLD (Jones et al., 1999; Liang et al., 1997). The analogous asparagine mutation in Arf6 (N48R) also inhibits PLD stimulation (Jovanovic et al., 2006) and these mutants have been used to discriminate PLD dependent and independent functions of Arf.

Rho

Rho family members were originally cloned from marine snails as homologues of Ras GTPases that shared approximately 35% sequence identity (Madaule and Axel, 1985). Today, over 20 Rho family members have been identified and the best characterized family members include RhoA, Rac1, and Cdc42 (Heasman and Ridley, 2008). Rho proteins are frequently activated following receptor stimulation and control

cytoskeletal dynamics such as formation of stress fibers, lamellipodia, and filopodia along with controlling membrane ruffling and cell polarity. Rho proteins have also been shown to regulate transcription of certain genes and promote cell proliferation, which underscores the importance of Rho proteins in cancer (Ellenbroek and Collard, 2007). Like other GTPases, Rho proteins are regulated by GEFs and GTPase-activating proteins (GAPs). However, Rho proteins are uniquely controlled by guanine nucleotide dissociation inhibitors (GDIs), which promote the inactive, GDP bound state, and prevent Rho-membrane association by sequestering their lipid moieties (Ellenbroek and Collard, 2007).

For some time, it was known that the combination of cytosol and non-hydrolyzable guanosine triphosphate (GTP) analogs such as GTPγS, stimulated PLD activity from human neutrophil membranes, implicating the participation of small molecular weight GTPases (Olson et al., 1991). By including GEFs and GDIs specific for Rho proteins in the neutrophil membrane PLD assay, investigators measured stimulation and inhibition, respectively of cytosol and GTPγS-stimulated PLD activity (Bowman et al., 1993; Kwak et al., 1995; Siddiqi et al., 1995). Although these experiments strongly suggested that the Rho family of GTPases mediated the cytosol-GTPγS stimulation of PLD activity in neutrophils, later studies with rat liver membranes confirmed the involvement of RhoA. Treatment with RhoGDI resulted in the loss of membrane-bound Rho and inhibition of the cytosol-GTPγS stimulation of rat liver PLD. Reconstitution with recombinant RhoA fully restored the PLD response following treatment with RhoGDI confirming that Rho proteins stimulate PLD activity (Malcolm et al., 1994). Additional, independent studies identified the stimulating factor as Rho due to sensitivity of PLD activity to the C3 toxin from *Clostridium botulinum,* which mediates the ADP-ribosylation of Rho proteins (Kuribara et al., 1995). In these studies, other Rho proteins such as

Rac1 and Cdc42 were partially able to rescue PLD activity following RhoA depletion or inactivation. Rho proteins were also identified as cytosolic factors capable of stimulating PLD activity from porcine brain (Brown et al., 1995; Singer et al., 1995).

Later studies using purified PLD1a and PLD1b demonstrated a direct stimulation of PLD1a and PLD1b activity by RhoA and associated family members Rac1 and Cdc42 (Hammond et al., 1997; Min et al., 1998; Walker and Brown, 2002) where RhoA stimulates PLD activity more than other family members. By contrast, PLD2 is relatively unresponsive to Rho family members (Colley et al., 1997b; Lopez et al., 1998; Sung et al., 1999a). Mutational analysis has mapped Rho-interaction site on PLD1 to the C-terminus (Sung, 1997; Yamazaki et al., 1999) and mutation of several non-conserved residues, K946A, V950A, R955A and K962A, inhibit RhoA binding and stimulation of PLD1 activity, potentially explaining the specificity for PLD1 versus PLD2 (Cai and Exton, 2001). Additionally, PKC, Arf, and RhoA are believed to bind distinct sites on PLD1 and modulate different kinetic parameters. Rho family members are thought to function primarily as binding activators and enhance substrate binding (Henage et al., 2006). As such, PKC, Arf, and Rho synergistically activate PLD1 *in vitro* (Hammond et al., 1997; Ohguchi et al., 1996) and most likely converge on PLD1 to precisely regulate activity *in vivo*. The mechanism by which Rho activates PLD *in vivo* is complicated by observation that Rho also stimulates PIP5K activity to increase PIP_2 levels (Chong et al., 1994). In fact, some studies suggest that the decrease in PLD activity following removal of Rho proteins might be due to a decrease in PIP_2 as the addition of PIP_2 is able to fully restore PLD activity in some circumstances (Schmidt et al., 1996). Thus, the role of Rho proteins *in vivo* might be to stimulate PIP_2 synthesis via an interaction with PIP5K and then to increase PLD substrate binding and subsequent activity as seen *in vitro*.

Ras and Ral

In addition to Arf and Rho, other small GTPases regulate PLD activity. Early investigations into the mechanisms of cellular transformation demonstrated that PLD activity was elevated in v-Ras and v-Src transformed fibroblasts compared to non-transformed cells (Carnero et al., 1994; Song et al., 1991). Maximal PLD activity in v-Src transformed fibroblasts required both cytosol and GTPγS and depletion of Ras from cytosol significantly decreased PLD activity, suggesting that Ras was required for the v-Src induced increase in PLD activity (Jiang et al., 1995a). Activated Ras has many downstream effectors including the guanine nucleotide dissociation stimulator for RalA, Ral-GDS (Hofer et al., 1994; Spaargaren and Bischoff, 1994). As such, later studies demonstrated that RalA was required for the v-Src induced increase in PLD activity since PLD formed a complex with RalA, but not Ras (Jiang et al., 1995b). The stimulatory properties of RalA are not direct, however, and experiments with purified proteins demonstrated that RalA does not directly stimulate PLD activity (Kim et al., 1998). However, RalA potentiates the stimulation of PLD1 by Arf proteins *in vitro* and Arf was found in a ternary complex with PLD1 and RalA when IP'ed from cell lysates (Kim et al., 1998). Indeed, inhibition of Arf GEFs in v-Src-transformed fibroblasts reduces PLD activity and suggests that Art is a key mediator of the v-Src-Ras-RalA-PLD transformation pathway (see "Functions of PLD in cancer")

Rheb

Regulation of the mammalian target of rapamycin (mTOR) pathways is complex and involves input signals from many sources. As discussed in the "Functions of PLD in cancer" section, PLD and PtdOH positively regulate mTOR (Fang et al., 2001). Rheb, a small Ras-like GTPase, also positively regulates mTOR activity through a mechanism that was thought to involve direct binding of Rheb to the mTOR complex (Long et al.,

2005). Rheb co-IPs with PLD1 from cell lysate and stimulates PLD1 activity *in vitro* (Sun et al., 2008) and the investigators proposed that Rheb positively regulates mTOR by regulating PLD1 activity. These *in vitro* experiments used IP'ed PLD1 from human cell lysate and bacterially expressed Rheb to demonstrate the regulation of PLD1 by Rheb. Follow up studies with highly purified PLD1 from insect cells did not reproduce the findings of the original paper suggesting that the regulation of PLD1 by Rheb likely involves an intermediate protein (unpublished data, Brown lab). Rheb is negatively regulated by the GAP activity of the tuberous sclerosis 2 protein (TSC2), a component of the tuberous sclerosis complex (Manning and Cantley, 2003). Cells deficient in TSC2 show higher PLD and mTOR activity (Sun et al., 2008; unpublished data), consistent with the idea of Rheb positively regulating mTOR through PLD.

Non G-protein/PKC regulators of PLD

Although PKC and small G-proteins appear to be the major regulators of PLD1 activity, a small number of additional PLD-stimulating factors have been identified although their role in the regulation of PLD is far less studied.

PKN. The protein kinase N (PKN) family of kinases are structurally related to PKC, yet are calcium-independent and not activated by phorbol esters (Morrice et al., 1994). These kinases are ubiquitously expressed (Kitagawa et al., 1995) and share many of the same intracellular locations as PLD1 (Kawamata et al., 1998). PKN is activated by GTP-bound RhoA and participates in cytoskeleton dynamics, cell migration, and tumor invasion (Watanabe et al., 1996). PKN directly stimulates PLD1 activity by binding to a region between amino acids 228 and 598 (Oishi, 2001). In addition, PtdOH, CL, and fatty acids are potent activators of PKN *in vitro* (Khan et al., 1994; Morrice et al., 1994). The activation of PKN and PLD by Rho and subsequent activation of PKN by

PtdOH presents an interesting positive feedback loop that likely explains some of the roles for PLD in regulating processes such as cytoskeleton rearrangement.

Cofilin. Cofilin proteins sever filamentous actin to generate free actin, an important process for cytoskeleton rearrangement (DesMarais et al., 2005). The activity of cofilin is regulated through a phosphorylation dependent mechanism where phosphorylation by LIM kinases results in cofilin inactivation and a reduction in actin severing resulting in actin polymerization. Rho and Rac/Cdc42 activate LIM kinases through Rho kinase and p21-activated protein kinase (PAK), respectively (Edwards and Gill, 1999; Kaibuchi et al., 1999; Sells and Chernoff, 1997). Phosphorylated cofilin stimulates PLD1 activity by directly binding to a region between the loop and CRIII (Han et al., 2007). The activation of PLD1 by phospho-cofilin underscores the importance of PLD1 in regulating cytoskeletal dynamics since Rho, Rac, and Cdc42 can directly bind and stimulate PLD1 and also indirectly activate PLD1 through a LIM kinase-cofilin pathway.

CtBP1. The C-terminal binding protein 1 (CtBP1) is a dual function protein that regulates gene transcription in the nucleus and also mediates membrane fission during intracellular trafficking events (Corda et al., 2006). CtBP1 specifically co-localizes with PLD1 following cell stimulation either with serum or EGF and purified CtBP1 was shown to stimulate PLD1 activity in an *in vitro* activity assay using purified proteins (Haga et al., 2009). Interestingly, CtBP1 stimulated PLD1 activity in an additive fashion with Arf and RhoA, suggesting that CtBP1 binds a distinct site on PLD1. Although further research is necessary, the membrane fission functions of CtBP1 might be mediated through PLD and PtdOH.

PLD inhibitory proteins

Most cells exhibit low basal PLD activity until stimulated by extracellular agonists or cellular stress (Zheng et al., 2006; Chapter III). *In vivo*, this stimulation most likely results from activation of stimulatory proteins and also inactivation of inhibitory proteins. As such, a number of PLD inhibitory factors have been purified over the years and a surprising number fall into the category of actin binding proteins or proteins involved with vesicular trafficking.

Synaptic vesicle proteins

Munc-18-1 is a syntaxin binding protein enriched in neurons and plays a critical role in synaptic vesicle exocytosis (Harrison et al., 1994; Wu et al., 1998). Munc-18-1 interacts directly with the PX domain of PLD1 and PLD2 and inhibits PLD activity *in vitro*. When overexpressed in cells, Munc-18-1 and PLD both co-localize and co-IP under basal conditions, but dissociate once stimulated with EGF when Munc-18-1 relocalizes from plasma membranes to the cytosol and no longer inhibits PLD activity (Lee et al., 2004). Therefore, cell-surface receptors may activate PLD by dissociating the Munc-18-1-PLD complex. In addition to Munc-18-1, clathrin assembly protein 3 (AP3) is another example of a trafficking protein known to regulate PLD activity. AP3 binds to clathrin triskelia and promotes assembly of clathrin-coated vesicles, which are dynamic organelles that participate in intracellular membrane trafficking (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988) including recycling of synaptic vesicles. Like Munc-18-1, AP3 is highly enriched in neuronal tissue (Ahle and Ungewickell, 1990; Keen, 1990) and was purified as a factor from rat brain cytosol capable of inhibiting purified PLD1 activity *in vitro* (Lee et al., 1997a). Even though AP3 is known to bind PIP_2 (Norris et al., 1995; Ye and Lafer, 1995), AP3 appears to bind and inhibit PLD1 in a PIP_2 dependent manner, suggesting a direct inhibition of PLD1 activity (Lee et al., 1997a). Like AP3,

amphiphysins are nerve terminal proteins that participate in clathrin-mediated synaptic vesicle endocytosis. Amphiphysins are believed to function in concert with dynamin to promote vesicle budding (Wigge and McMahon, 1998). As with AP3, amphiphysin I and II were purified from rat brain cytosol as factors capable of inhibiting purified PLD1 and PLD2 activity *in vitro* and inhibit PMA-stimulated PLD activity when overexpressed in cells (Lee et al., 2000). Although the exact physiological relevance of PLD inhibition by these synaptic vesicle proteins is unknown, they most likely function to terminate PtdOH generation during the early stages of vesicle formation.

Actin binding proteins

The role of PLD and PtdOH in regulating cytoskeletal dynamics is complicated and likely involves many upstream and downstream regulators. A number of actin binding proteins, and actin itself, have been identified as inhibitors of PLD activity. Stimulation of cytosol with GTPγS results in the association of PLD with detergent insoluble fractions, which are highly enriched in actin and actin-binding proteins (Hodgkin et al., 1999; Iyer and Kusner, 1999). Actin exists in either monomeric (G-actin) or filamentous (F-actin) forms and both have been shown to bind PLD *in vitro* (Kusner et al., 2002). Monomeric G-actin stimulates PLD activity whereas F-actin inhibits PLD activity (Kusner et al., 2002; Lee, 2001). The site of interaction between actin and PLD2 was mapped to a region between CRIII and CRIV for PLD2 (Lee, 2001). Additionally, actin binding proteins such as fodrin (Lukowski et al., 1996), spectrin (Lukowski et al., 1998), α-actinin, and gelsolin (Banno et al., 1999) have also been shown to directly inhibit PLD activity. These actin-binding proteins connect microfilaments to give the cytoskeleton its characteristic shape. The exact physiological function of PLD inhibition by F-actin and actin binding proteins is not known, but the several lines of evidence suggest that PLD activity promotes the early stages of actin polymerization. First,

inactive-cofilin promotes actin polymerization and PLD activity. Second, monomeric actin stimulates PLD activity whereas filamentous actin inhibits PLD activity. Finally, interactions with proteins that hold the established actin cytoskeleton together inhibit PLD activity. Along with PLD activation by Rho family members, these data strongly implicate a role for PLD in cytoskeletal remodeling.

Miscellaneous PLD inhibitors

In addition to the vesicular and actin-associated PLD inhibitors, several proteins have been identified as PLD inhibitors that do not fit into broad categories. Aldolase is a glycolytic enzyme that mediates the reversible cleavage of fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Aldolase was identified as a cytosolic factor that directly inhibits PLD2. Mutational analysis narrowed the site of interaction to the PH domain of PLD2 (Kim et al., 2002). The physiological function of the aldolase-PLD2 interaction is unknown, but this interaction might contribute to the role of PLD in regulating cellular bioenergetics (Chapter II). Another protein known to inhibit PLD is the $G\beta\gamma$ subunit from heterotrimeric G-proteins. GPCR stimulation results in dissociation of $G\beta\gamma$ from $G\alpha$ and $G\beta\gamma$ regulates downstream effector molecules containing PH domains such as GPCR kinases (GRKs) (Carman et al., 2000) and PLCβ (Wang et al., 2000). Recombinant $G\beta\gamma$ inhibits PLD1 or PLD2 activity *in vitro*, presumably through an interaction with PH domain since an N-terminal PLD truncation mutant was resistant to $G\beta\gamma$ inhibition (Preininger et al., 2006). Thus, $G\beta\gamma$ inhibition of PLD serves as one of the ways PLD signaling is terminated following GPCR stimulation.

Finally, two neuronal proteins have been identified as PLD2 inhibitors. The first protein, α-synuclein, is a small, highly conserved protein of unknown function that is

highly enriched in the brain presynaptic terminals. α-synuclein is a major component of Lewy bodies and Lewy neuritis, which are neuropathological hallmarks of familial and sporadic Parkinson's disease (Spillantini et al., 1997). α-synuclein was purified as a factor capable of inhibiting purified PLD2 activity *in vitro* (Jenco et al., 1998). This observation was later hypothesized as a mechanism by which neurons are protected from the toxicity associated with elevated PLD activity (Gorbatyuk et al., 2010). In these studies, PLD2 was purified by an immunoaffinity technique and no gels of purity were shown. A Later study with highly purified PLD2 was unable to replicate the original findings (Rappley et al., 2009), suggesting the presence of an intermediate protein in the original paper. Regardless of the interaction between α-synuclein and PLD2, no associations have been made *in vivo* to support a role for PLD2 in the pathophysiology of α-synuclein (Ahn et al, 2002). Therefore, the relevance and nature of this interaction is still a matter for investigation. The second neuronal protein capable of directly inhibiting PLD2 is collapsin response mediator protein-2 (CRMP-2). CRMP-2 is a critical component of neuron outgrowth during development and is believed to participate in early stages of Alzheimer's disease (Hensley et al., 2011). Like α-synuclein, CRMP-2 was purified as a cytosolic brain factor capable of inhibiting purified PLD2 *in vitro* and is believed to directly inhibit activity by binding to the N-terminus (Lee et al., 2002). Future studies will no doubt shed light on the role of PLD2 pathologies such as Alzheimer's disease. A table of PLD regulatory proteins is presented below (Table 1).

Table 1. PLD regulatory proteins

Protein Name	Isoform	Mechanism	Interaction site	Reference (s)
Activators				
PKC α,β1,β2	PLD1	Protein-protein interaction	N-terminal (AA 50-115) C-terminal (AA 325-582)	(Conricode et al., 1992; Lee et al., 1997b; Siddiqi et al., 2000; Conricode et al., 1994; Ohguchi et al., 1996; Min et al., 1998; Hammond et al., 1997, Sung et al., 1999b; Park et al., 1998; Zhang et al., 1999; Park et al., 1998)
Arf	PLD1	Protein-protein interaction	Unknown	(Brown et al., 1993; Cockcroft et al., 1994; Hammond et al., 1997; Lopez et al., 1998; Min et al., 1998)
RhoA family	PLD1	Protein-protein interaction	C-terminal (K946A, V950A, R955A, K962A)	(Olson et al., 1991; Bowman et al., 1993; Kwak et al., 1995; Siddiqi et al., 1995; Malcolm et al., 1994; Brown et al., 1995; Singer et al., 1995; Hammond et al., 1997, Sung et al., 1997; Yamazaki et al., 1999; Cai and Exton, 2001)
PKN	PLD1	Protein-protein interaction	AA 228-598	(Oishi et al., 2001)
Rheb	PLD1	Protein-protein interaction	Unknown	(Sun et al., 2008)
Ras	PLD1	Indirect protein-protein interaction	Unknown	(Jiang et al., 1995a)
RalA	PLD1	Indirect protein-protein interaction	Unknown	(Jiang et al., 1995b; Kim et al., 1998)
Cofilin	PLD1	Protein-protein interaction	Region between loop and CRIII	(Han et al., 2007)
CtBP1/BARS	PLD1	Protein-protein interaction	Unknown	(Haga et al., 2009)
AMPK	PLD1	Phosphorylation	S505	(Kim et al., 2010)
p90 RSK	PLD1	Phosphorylation	T147	(Zeniou-Meyer et al., 2008)
Cdk5	PLD2	Phosphorylation	S134	(Lee et al., 2008)
Grb2	PLD2	Protein-protein interaction	Y169/Y179	(Di Fulvio et al., 2006)
PKCδ	PLD2	Phosphorylation	S566	(Han et al., 2002a; Chae et al., 2010)
Inhibitors				
AP3	PLD1	Protein-protein interaction	Unknown	(Lee et al., 1997a)
Aldolase	PLD2	Protein-protein interaction	PH domain	(Kim et al., 2002)
α-actinin	PLD2	Protein-protein interaction	Unknown	(Park et al., 2000)
CRMP-2	PLD2	Protein-protein interaction	N-terminus	(Lee et al., 2002)
Munc-18-1	PLD1/PLD2	Protein-protein interaction	PX domains	(Lee et al., 2004)
Amphiphysin I and II	PLD1/PLD2	Protein-protein interaction	Unknown	(Lee et al., 2000)
F-actin	PLD1/PLD2	Protein-protein interaction	Region between CRIII and CRIV	(Kusner et al., 2002; Lee et al., 2001)
Gelsolin	PLD1/PLD2	PIP_2 binding	Unknown	(Banno et al., 1999)
Gβγ	PLD1/PLD2	Protein-protein interaction	PH domains	(Preininger et al., 2006)
PKCα	PLD1/PLD2	Phosphorylation	PLD1 (S2, T147, S561) PLD2 (S134, S146, S243, T72, T99, T100, T252)	(Kim et al., 1999a; Chen and Exton, 2005)
Fodrin	Not determined	PIP_2 binding	Unknown	(Lukowski et al., 1996)
Spectrin	Not determined	PIP_2 binding	Unknown	(Lukowski et al., 1996)

Cell-surface receptor regulation of PLD

Receptor Tyrosine Kinases

Ligand binding to cell surface receptors is known to stimulate PLD activity in many cell lines and tissues. EGF stimulates PLD activity (Fisher et al., 1991) and this pathway has been used as a model system for characterizing the intermediate signaling events between receptor tyrosine kinases (RTK) and PLD over the past 25 years. EGF binding to the EGFR results in receptor dimerization and autophosphorylation of several tyrosine residues within the cytoplasmic tails via an intrinsic kinase activity (Schlessinger and Ullrich, 1992). These phosphorylated tyrosine residues create high affinity binding sites for proteins containing Src-homology 2 (SH2) domains such as PLC-γ1, Grb2, the p85 subunit of PI3K, Src, SHP-2, and Bruton's tyrosine kinase, which mediate a variety of signaling events including those that regulate DNA synthesis, cell division and proliferation, cytoskeletal rearrangements, and a variety of other biological functions (Yaffe, 2002). As such, several mechanisms of PLD activation by EGF have been described.

Early studies of PC hydrolysis or phosphatidyl alcohol production following EGF stimulation in intact tells did not discriminate between PLD1 and PLD2 activation, although later studies have shown that both isoforms couple to EGFR (Slaaby et al., 1998). The most-studied EGFR-PLD signaling pathway begins with activation of PLC-γ1 by binding to activated EGFR via its SH2 domain (Anderson et al., 1990; Margolis et al., 1990). Activation of PLC-γ1 results in the rapid hydrolysis of PIP_2 into inositol triphosphate (IP_3) and DAG. IP_3 increases intracellular calcium levels, which together with DAG, activates PKC (Rana and Hokin, 1990). As discussed in the "Regulation of PLD" section, PKC is a well-established activator of PLD and multiple studies have

demonstrated an EGFR-PLC-γ1-PKC axis for PLD activation. By using small molecule PKC inhibitors or by down-regulating PKC levels with prolonged phorbol ester treatment, multiple groups have shown decreased EGF-stimulated PLD activation following inactivation of PKC in various cell types (Voss et al., 1999; Yeo and Exton, 1995). Later studies using mouse embryonic fibroblasts demonstrated compromised EGF stimulation of PLD activity in PLC-γ1 null cells versus wild-type cells, supporting the requirement of PLC-γ1 and PKC for EGF stimulation of PLD activity (Hess et al., 1998). Although PKC is believed to primarily activate the PLD1 isoform (Colley et al., 1997b; Sung et al., 1999b), the PX domain of PLD2 interacts with the SH3 domain of PLC-γ1 following EGF stimulation and is believed to increase PLC-γ1 mediated hydrolysis of PIP_2 (Jang, 2003). When the PLD2-PLC-γ1 interaction is disrupted by mutating specific proline residues, EGF no longer stimulates PLD2 activity, indicating a complex mode of PLD2 regulation by PLC-γ1 (Jang, 2003).

Although EGFR activation stimulates PLD activity through a PLC-γ1-PKC axis in many cell types, EGFR activation does not always stimulate PIP_2 hydrolysis and PKC activation in other cell lines (Cook and Wakelam, 1992; Hess et al., 1997). Alternative EGFR-PLD coupling mechanisms have been described and usually involve activation of small GTPases. Ras participates in a complex regulatory mechanism with RTK-stimulated PLD. The first step of the canonical Ras activation sequence involves recruitment and activation of Sos, which stimulates GDP-GTP exchange on Ras and allows Ras to activate downstream effectors (Schlessinger, 2000). GEFs for the small GTPase RalA are stimulated by activated Ras (Matsubara et al., 1999) and RalA has been implicated in the activation of PLD. RalA interacts with PLD1 (Luo et al., 1997) and is believed to enhance PLD catalytic activity in the presence of other activators such as Arf GTPase (Kim et al., 1998). As such, PLD stimulation by EGFR requires Ras-

activated RalA in multiple cell lines (Lu et al., 2000; Voss et al., 1999), although the intermediate signaling proteins between RalA and PLD are not fully characterized. In addition to RalA, Ras is known to directly activate the p110 subunit of PI3K by binding to a Ras binding domain (RBD) (Rodriguez-Viciana et al., 1996). The product of PI3K, PIP_3, can recruit and activate PLD1 following RTK stimulation via the PLD-PX domain (Lee et al., 2005; Standaert et al., 1996). Although speculative, Ras activation of PI3K could also influence PLD activity through a PIP_3-dependent mechanism.

By contrast, PLD and PtdOH influence EGF-stimulated Ras activation by regulating membrane recruitment and activation of Sos. The adaptor molecule Grb2 contains two SH3 domains and one SH2 domain. Receptor stimulation recruits Grb2 via an interaction with the SH2 domain. Once receptor bound, Grb2 binds Sos via one of the SH3 domains and this has been a well-established mechanism for Sos recruitment (Schlessinger, 2000). PLD2 binds the other SH3 domain of Grb2 and that this interaction was necessary for EGF stimulation of PLD2 activity (Di Fulvio et al., 2006). Interestingly, PLD-generated PtdOH also directly binds and recruits Sos to membranes following EGFR activation (Zhao et al., 2007). Thus, a potential feed-forward mechanism emerges where PLD activates Ras which leads to activation of downstream effectors such as RalA, further stimulating PLD activity. The regulation of Ras signaling by PLD has important clinical implications and is further discussed in the “Functions of PLD in cancer” section.

In addition to Ras, Arf proteins also participate in PLD1 and PLD2 activation following RTK stimulation. RTK activation of PI3K recruits certain GEFs for Arf proteins (Venkateswarlu et al., 1998) and results in the formation of an active ArfGEF-RTK complex (Li et al., 2003). Studies using Brefeldin A, an inhibitor of certain ArfGEFs (Peyroche et al., 1999), demonstrated that the ArfGEF ARNO and Arf1 are required for PLD1 activation following stimulation of the insulin receptor in rat fibroblasts (Li et al.,

2003; Shome et al., 1997). The requirement of Arf in insulin signaling appears to be cell line dependent as PLD activation by insulin does not require Arf in CHO-T cells (Emoto et al., 2000). Additionally, Arf4 was identified as an EGFR binding partner in a yeast two-hybrid screen that interacts with EGFR upon agonist stimulation (Kim et al., 2003b). Co-expression of Arf4 with PLD2 but not PLD1 resulted in a substantial increase in EGF stimulated PLD2 activity compared to the expression of PLD2 alone (Kim et al., 2003b). Although further studies are required to delineate the role of Arf proteins in RTK-PLD activation, these current studies suggest that Arf proteins may play non-redundant roles in regulating PLD1 vs. PLD2.

The Rho family of small GTPases (Rho/Rac/Cdc42) also regulates RTK stimulation of PLD activity in some cell systems. Like Ras and Arf, Rho GTPases are usually activated by upstream GEFs through a variety of mechanisms (Buchsbaum, 2007). In fibroblasts, Rac1 and RhoA were required for PLD activation by PDGF and EGF, respectively (Hess et al., 1997). However, RTK stimulation of PLD in vascular smooth muscle cells requires Arf proteins but not Rho proteins (Shome et al., 2000). Rho proteins are particularly important for regulating cytoskeleton rearrangements needed for processes such as stress fiber and lamellipodia formation in motile cells (Nobes and Hall, 1995). The role of RTK signaling to PLD through Rho family proteins may thus be dependent on the motile nature of the cell.

Recent studies have elucidated new regulators of RTK-PLD signaling in addition to the “classic” PLD inhibitors previously discussed. Cyclin dependent kinase 5 (Cdk5) is a multifunctional serine/threonine kinase that mediates a variety of signaling events including exocytosis of insulin in pancreatic β-cells (Lilja et al., 2004). Upon stimulation with EGF in rat insulinoma cells, Cdk5 phosphorylates PLD2 at serine 134 and this phosphorylation event was required for EGF stimulated PLD activity and subsequent release of insulin from these cells (Lee et al., 2008). Future studies should determine if

the phosphorylation of PLD2 by Cdk5 is required for RTK-mediated function in other cell types. The multitude of pathways in which RTKs stimulate PLD activity underscores the importance of PLD and PtdOH in mediating the biological effects of RTK ligands.

G-protein coupled receptors

GPCRs represent another large class of cell surface receptors that frequently lead to PLD activation. Under inactive conditions, GPCRs bind different classes of heterotrimeric G-proteins composed of an alpha subunit and a dimer composed of beta and gamma subunits. Ligand binding at the extracellular face of the GPCR leads to a conformational change in the receptor that promotes GDP to GTP exchange on the α subunit and GTP-bound Gα then dissociates from $\beta\gamma$ (Oldham and Hamm, 2008). Each class of activated Gα subunit is associated with a distinct cellular signaling pathway. The $G\alpha_s$ G-proteins activate adenylyl cyclase (AC) to increase intracellular levels of the second messenger cyclic AMP (cAMP), which activates protein kinase A (PKA). The $G\alpha_{i/o}$ G-proteins are associated with a decrease in AC activity, however, the liberated G$\beta\gamma$ dimer has AC-independent roles such as recruiting and activating proteins with PH domains (Touhara et al., 1994). $G\alpha_{q/11}$ G-proteins stimulate PLC activity, which subsequently leads to activation of PKC and downstream effectors (McCudden et al., 2005). Finally, $G\alpha_{12/13}$ proteins are associated with activation of Rho proteins and effectors by activating Rho GEFs (Kozasa et al., 1998). The list of GPCR ligands that activates PLD is quite extensive and GPCRs of all classes have been reported to activate PLD (Exton, 1999). Therefore, the known mechanisms of PLD activation by each class of Gα G-protein are discussed in the following section.

Once activated, $G\alpha_q$ binds and recruits PLC-β to membranes where it hydrolyzes PIP_2 to generate IP_3 and DAG, leading to activation of PKC (Rhee, 2001). Several lines

of evidence suggest that PKC mediates the activation of PLD by $G\alpha_q$. Cells that express constitutively active $G\alpha_q$ have elevated PLD activity (Plonk et al., 1998). Co-expression of a PKC-resistant PLD mutant significantly reduces the fold-activation of PLD activity due to constitutively active $G\alpha_q$, suggesting that PKC mediates $G\alpha_q$ activation of PLD (Xie et al., 2002b). Likewise, stimulation of a $G\alpha_q$ coupled receptor failed to stimulate another PKC-resistant PLD mutant. This mutant was responsive to non-$G\alpha_q$ GPCR stimuli, confirming proper protein folding and underscoring the importance of PKC in mediating $G\alpha_q$-PLD signaling (Zhang et al., 1999). Small-molecule PKC inhibitors block PLD activation by a number of GPCR agonists including bradykinin (Pyne and Pyne, 1995), thrombin (Vasta et al., 1998), and S1P (Meacci et al., 1999) and overexpression of PKC-β is known to potentiate the PLD response to endothelin-1 stimulation in Rat6 fibroblasts (Pai et al., 1991). Similarly, downregulation of PKC with prolonged phorbol ester treatment inhibits bradykinin stimulation of PLD in SF3271 fibroblasts (Clark and Murray, 1995). Data from our lab suggest that the combination knockdown of PKCα and PKCβ significantly decreases M1-muscarinic receptor stimulation of PLD in HEK293 cells (data not shown), confirming the importance of PKC in mediating $G\alpha_q$ signaling to PLD. However, many $G\alpha_q$-coupled GPCRs couple to additional G-proteins including members of the $G\alpha_{12/13}$ family.

$G\alpha_{12/13}$ G-proteins have long been known to stimulate cytoskeletal rearrangements through activation of the Rho family of proteins and some GPCRs that predominantly couple to $G\alpha_q$ also couple to $G\alpha_{12/13}$. For example, GPCRs such as the M3 muscarinic receptor stimulate PLD in a PKC-independent manner in certain cell systems. Expression of a dominant negative RhoA construct or treatment with C3 exoenzyme inhibits M3 stimulation of PLD, suggesting RhoA participates in M3 activation of PLD (Mitchell et al., 1998; Schmidt et al., 1996). Likewise, expression of a

RhoA-resistant PLD mutant is less responsive to M3 stimulation (Du et al., 2000). C3 exoenzyme treatment inhibits the PLD response to other GPCR agonists such as bradykinin (Meacci et al., 2003) and S1P (Meacci et al., 2003). Expression of constitutively active $G\alpha_{13}$, like $G\alpha_q$, stimulates PLD activity. This active $G\alpha_{13}$ mutant stimulates PKC-resistant PLD constructs, suggesting that $G\alpha_{13}$ stimulates PLD independently of PKC. C3 exoenzyme treatment inhibits $G\alpha_{13}$ activation of PLD (Plonk et al., 1998; Xie et al., 2002a), suggesting that RhoA mediates $G\alpha_{13}$ stimulation of PLD. In addition to a direct protein-protein interaction between RhoA and PLD, several other activation mechanisms have been proposed. RhoA is known to stimulate synthesis of PIP_2 (Chong et al., 1994) and exogenous addition of PIP_2 following RhoA inactivation is known to restore PLD activity (Schmidt et al., 1996). Besides direct stimulation of PLD activity and increasing PIP_2 levels, a recent study suggested that LIM1 kinase, a RhoA effector, stimulates PLD activity following M3 stimulation. As discussed previously, LIM1 kinase phosphorylates cofilin, and phospho-cofilin stimulates PLD activity downstream of M3 activation (Han et al., 2007). Phospho-cofilin depletion had no effect on PMA stimulated PLD activity, suggesting a RhoA dependent mechanism. On the other hand, several studies suggest that RhoA and PKC synergize to stimulate PLD downstream of GPCRs. For example, C3 exoenzyme reduces PLD activity following S1P stimulation in C212 myoblasts, but PLD activity is further reduced following with treatment of PKC inhibitors (Meacci et al., 2001). Additionally, overexpression of RhoA partially rescues M3-mediated activation of PKC-resistant PLD and together these results suggest that both RhoA and PKC can mediate signals from the same GPCR to PLD (Zhang et al., 1999).

The signaling pathways from $G\alpha_s$ coupled receptors to PLD remains somewhat elusive. Stimulation of $G\alpha_s$-coupled GPCRs or treatment of cells with AC-activating

compounds such as forskolin and dibutyryl-cAMP results in PLD activation (Ginsberg et al., 1997). Under these conditions, treatment of cells with PKA inhibitors reduces PLD activity, suggesting that PKA somehow mediates PLD activation (Yoon et al., 2005). Additionally, transfection of DN-Src and DN-Ras also decreases PLD activation downstream of AC, suggesting that Src and Ras participate in $G\alpha_s$ coupling to PLD (Yoon et al., 2005). Epac1 is a cAMP-activated GEF for small G-proteins such as Rap1. Recent studies have shown that depletion of Epac1 inhibits $G\alpha_s$ signaling to PLD. Epac1 also promotes GDP to GTP exchange on R-Ras and this AC-Epac1-R-Ras pathway has been implicated in PLD activation (López de Jesús et al., 2006)

Several GPCRs activate PLD in a pertussis toxin sensistive manner, suggesting that $G\alpha_{i/o}$ proteins also stimulate PLD activity. For example, stimulation with fMLP in neutrophils or S1P in A549 lung adenocarcinoma cells and in human airway epithelial cells results in pertussis toxin sensitive stimulation of PLD (Fensome et al., 1998; Ghelli et al., 2002; Meacci et al., 2003). Additionally, known $G\alpha_{i/o}$ receptors such as M2 and M4 muscarinic receptors stimulate PLD activity in HEK293 cells (Sandmann et al., 1991). The pathways by which $G\alpha_{i/o}$ stimulate PLD are relatively uncharacterized, but is thought to require the activity of Arf and Rho (Fensome et al., 1998) and participation by tyrosine kinases such as Src (Ghelli et al., 2002). Likewise, Arf activity is required for stimulation of PLD activity by pertussin toxin insensitive GPCRs (Mitchell et al., 2003; Mitchell et al., 1998; Rumenapp et al., 1995). Liberated $G\beta\gamma$ subunits are known to activate certain PI3Ks and increase local concentrations of PIP_3 following receptor activation (Stephens et al., 1997). Thus, one potential mechanism for Arf activation appears to be recruitment of PI3K-dependent ArfGEFs downstream of $G\beta\gamma$ (Touhara et al., 1994). Whether other mechanisms besides $G\beta\gamma$ stimulation of GEFs mediate $G\alpha_{i/o}$ stimulation of PLD should be the subject of future investigations. Additionally, Arf is known to participate in PLD

activation by $G\alpha_q$ and $G\alpha_s$ – coupled receptors (Mitchell et al, 2003; Thibault et al., 2002).

The PtdOH derived from GPCR-stimulated PLD may function to terminate GPCR signaling by regulating the activity of proteins required for GPCR inactivation. GRKs phosphorylate GPCRs and create a high affinity binding site for arrestin proteins, which uncouple GPCRs from G proteins and promote receptor internalization and desensitization (Moore et al., 2007). PtdOH, along with phosphatidylserine (PS), CL, and PI, are stimulators of GRK2/3 activity *in vitro* as determined by measuring phosphorylation of GPCRs in micelles containing phospholipids and purified GPCRs incubated with purified GRK. Kinase activity towards muscarinic (DebBurman et al., 1995) and adrenergic (Onorato et al., 1995) receptors was much higher in the presence of PtdOH, suggesting that PLD may promote GPCR internalization and desensitization through GRKs.

Termination of AC signaling requires the activity of cyclic nucleotide phosphodiesterases (PDE) that hydrolyze 3', 5'- cyclic nucleotides to a nucleoside 5'-monophosphate. Several families of cyclic-AMP specific PDEs have been identified that are responsible for terminating signaling events mediated by this important second messenger (Conti and Beavo, 2007). As such, multiple variants of the cAMP-specific PDE4 family are regulated by phosphatidic acid. Screening recombinant variants of PDE4 enzymes for PtdOH sensitivity revealed that PDE4A5, PDE4D3, and PDE4EB1 were all stimulated by PtdOH in an *in vitro* enzyme assay (Némoz et al., 1997). PDE4D3 was shown to bind PtdOH directly and the binding site was mapped to a region enriched in basic amino acids from 31-59 (Grange 2002). Another PDE4 family member, PDE4A1 is not activated *in vitro* by PtdOH (Némoz et al., 1997), but requires an interaction with PtdOH at its amino terminus for proper membrane localization (Baillie et al., 2002).

Thus, by activating PDEs, PtdOH may function to terminate $G\alpha_s$ signaling by promoting metabolism of the cAMP second messenger.

Functions of PLD in cancer

Over the course of nearly 40 years of research, PLD enzymes have been implicated in a variety of pathologies ranging from neurodegenerative diseases (Lindsley and Brown, 2012) to blood disorders (Elvers et al., 2010). The best characterized, disease relevant roles for PLD relate to the requirement of PLD activity for oncogenesis and cancer progression. Early indications of the importance of PLD and PtdOH in cancer came from observations that cells transformed by viral oncogenes such as v-Src (Song et al., 1991), v-Fps (Jiang et al., 1994) v-Ras (Carnero et al., 1994; Jiang et al., 1995a), and v-Raf (Frankel et al., 1999) all showed elevated PLD activity relative to non-transformed control cells. Furthermore, PLD mRNA and protein analyses from tumors of breast (Noh et al., 2000; Uchida et al., 1997), renal (Zhao et al., 2000), colorectal (Yoshida et al., 1998), gastric (Uchida et al., 1999), thyroid (Kim et al., 2008), and brain (Park et al., 2009) origin show elevated PLD1 and/or PLD2 expression relative to normal surrounding tissue. As such, overexpression of PLD isoforms is associated with enhanced tumorigenesis in cultured cells as measured by increased anchorage-independent growth (Ahn et al., 2003; Min, 2001), the most important measure of tumorigenicity (Shin et al., 1975). Recent genomic analyses of human cancers have revealed several unique PLD mutations in breast (Wood et al., 2007), stomach (Zang et al., 2012), and brain (Molenaar et al., 2012; Pugh et al., 2012) cancers although most of the reported mutations remain to be functionally characterized. Due to the common upregulation of PLD expression and activity along with the growing list of PLD mutations, researchers have long assumed that PLD and PtdOH confer growth advantages to transformed cells.

Definitive characterization of the molecular mechanisms behind these growth advantages has been difficult due to the lack of specific, small-molecule inhibitors. The three most common techniques for studying PLD function have been the use of primary alcohols to produce phosphatidyl alcohols at the expense of PtdOH via the PLD transphosphatidylation reaction (Yang et al., 1967), RNA interference (RNAi) to silence the expression of PLD mRNA, and expression of catalytically inactive PLD mutant proteins that function in a dominant negative manner. Each technique has its own set of flaws including non-specific enzyme inhibition, incomplete knockdown of target proteins, and retention of lipase-independent activities. The recent development of isoform-selective PLD inhibitors (Lavieri et al., 2010; Scott et al., 2009) and PLD knockout mice (Elvers et al., 2010; Oliveira et al., 2010) are now being integrated into the arsenal of tools to study PLD function and will certainly assist in defining the exact roles of PLD in cancer.

The path to malignancy requires several unique steps. Hanahan and Weinberg have proposed eight “hallmarks” of cancer that comprise eight biological capabilities acquired during the development of human tumors. These include sustaining proliferative signaling, evading growth suppression, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, avoiding immune destruction, and deregulating cellular energetics (Hanahan and Weinberg, 2011). Interestingly, PLD has been implicated in almost every hallmark of cancer and the following discussion will highlight these various cancer-related PLD functions.

Sustaining proliferative signaling

In the absence of growth factors, cells enter a state of quiescence and no longer proliferate. This state of cellular quiescence is usually reached following terminal differentiation and is critical for maintaining proper tissue function and structure. Cancer

cells acquire mutations that allow abnormal proliferation in the absence of normal growth signals. Mitogenic signals, or signals that allow passage through cell cycle checkpoints, are usually mediated by cell surface receptors such as EGFR and their downstream effectors including the mitogen activated protein kinase (MAPK) pathways and PI3K/Akt/mTOR pathways. Mutated or overexpressed growth factors receptors including the PDGFR and EGFR are commonly observed in malignancies such as glioblastoma (GBM) and breast cancer, respectively (Shih and Holland, 2006; Slamon et al., 1987). Cancer cells also upregulate and secrete growth factors to stimulate growth in an autocrine fashion (Sporn and Roberts, 1985). As discussed previously, PLD is frequently activated as a consequence of receptor stimulation and participates in the pathways of cellular proliferation. Early evidence that PLD might directly regulate cell proliferation came from observations that exogenously added PtdOH or highly active bacterial PLD increased levels of c-Fos and c-Myc transcription factors, and stimulated thymidine uptake, a marker of DNA synthesis and cell proliferation, when added to cultured cells (Moolenaar et al., 1986). Since these early studies, PLD has been shown to participate in several cell proliferation pathways.

Mitogen activated protein kinase

One of the most commonly deregulated proliferative pathways in human cancers is the MAPK pathway. Growth factors, hormones, and chemokines activate the MAPK pathway by signaling through their cognate receptors and initiating a series of signaling events that leads to activation of multiple protein kinases (Shaw and Cantley, 2006). Under the canonical activation sequence, receptor activation recruits exchange factors for Ras to the membrane, and stimulates GDP to GTP exchange to activate Ras. Raf kinases subsequently bind activated Ras at the membrane and phosphorylate an intermediate kinase, generally termed mitogen-activated protein kinase kinase (MEK). Activated MEK

phosphorylates and activates a terminal MAPK termed extracellular-signal regulated kinases 1 and 2 (ERK1/2) (De Luca et al., 2012). Activated ERK1/2 upregulates expression of many genes important for cell cycle progression, metabolism, and proliferation by activating a plethora of transcription factors such as NFAT, Elk-1, MEF2, c-Fos, c-Myc, and STAT3 (Roux and Blenis, 2004). ERK1/2 can regulate protein translation by activating p90 ribosomal S6 kinase, MAPK-activated protein kinases, and MAPK-interacting kinases, which are known to directly regulate the activity of ribosomal translation machinery (Chen et al., 2001). Additionally, ERK1/2 increases expression of anti-apoptotic genes and Raf can directly inhibit the apoptotic machinery at the mitochondrial membrane, independently of ERK (Alejandro and Johnson 2008). PLD and PtdOH have been intricately linked to multiple steps within the MAPK pathway and overexpression of PLD has been linked to upregulated ERK activity as measured by increased gene transcription downstream of ERK-activated transcription factors such as STAT3 (Choi and Han, 2012).

Ras was one of the earliest identified oncogenes, first discovered as a viral protein capable of inducing sarcoma in rats (Malumbres and Barbacid, 2003). Around 30% of human tumors contain mutations in Ras genes that result in constitutive Ras activation (Dunn et al., 2005). A growing body of literature suggests that PLD and PtdOH directly regulate Ras activation through several mechanisms. A well-established Ras activation pathway involves recruitment of the adaptor protein Grb2 to phosphorylated tyrosine residues on RTKs via its SH2 domain (Lowenstein et al., 1992). Grb2 contains two SH3 domains (Matuoka et al., 1992) that bind proteins with proline-rich motifs, such as the Ras GEF Sos (Chardin et al., 1993; Simon et al., 1991). Ras family members undergo farnesylation or geranylgeranylation that results in constitutive membrane association. Thus, formation of the RTK/Grb2/Sos complex allows GDP to GTP exchange and activation of membrane-associated Ras. In addition to RTKs, Grb2 can

bind PLD2 via the Grb2 SH2 domain at PLD2-Y169 and Y179 (Di Fulvio et al., 2006). The PLD2-Grb2 complex recruits Sos and stimulates activation of Ras and MAPK pathways (Di Fulvio et al., 2006; 2008) in a manner that does not require phospholipase activity. Furthermore, the PX domain of PLD2 has been shown to act as a GEF for Ras directly (Henkels et al., 2013b), providing an alternative route for lipase independent Ras activation. On the other hand, PtdOH directly regulates Ras activation by serving as a recruitment signal for the Sos exchange factor. Sos contains a PH domain that binds PtdOH, and residues H475 and R479 mediate this interaction. When PtdOH-binding residues are mutated, SOS is not longer recruited to the plasma membrane following EGFR stimulation. PLD2 was found to co-localize with SOS and genetic silencing of PLD2 prevented the EGF-stimulated recruitment of SOS to the membrane and subsequent activation of Ras (Zhao et al., 2007). Additionally, PtdOH can inhibit the activity of neurofibromin 1 (NF1), a Ras GAP, *in vitro*. NF1 was identified as one the earliest tumor suppressor genes (Cichowski and Jacks, 2001) and while the *in vivo* relevance of PtdOH inhibition of NF1 remains to be established, upregulated PLD activity could potentially activate Ras by inhibiting NF1. The diverse mechanisms by which PLD can regulate Ras activity certainly underscore the importance of PLD and PtdOH for Ras activation.

On the other hand, Ras can activate PLD via a mechanism involving Ral-GDS, RalA, and Arf and the importance of PLD and PtdOH in Ras-mediated tumorigenesis was demonstrated in an elegant study where the introduction of dominant negative PLD into rat fibroblasts blocked the transforming ability of constitutively active Ras. When the Ras-transformed fibroblasts were injected into immunocompromised mice, fibroblasts co-expressing DN-PLD failed to form tumors. However, when PtdOH was delivered to the mice via osmotic pump, Ras-transformed fibroblasts expressing DN-PLD formed tumors similarly to Ras-transformed fibroblasts that did not express DN-PLD (Buchanan

et al., 2005). Other studies have also demonstrated the importance of PLD as an oncogenic Ras signal-transduction molecule. One of the negative regulators of ERK is a small, 15kDa protein known as protein enriched in astrocytes, or PEA15. Originally identified as a substrate for PKC in astrocytes (Araujo et al., 1993), it was later identified in yeast-two hybrid screen as a binding partner of PLD1 (Zhang et al., 2000). PEA15 positively regulates PLD activity, potentially by acting as a chaperone protein to increase protein stability and decrease degradation (Zhao et al., 2000). PEA15 also directly inhibits ERK by sequestration in the cytoplasm and blocking ERK nuclear import (Formstecher et al., 2001; Pastorino et al., 2010). On the other hand, when PEA15 is phosphorylated at S116 by Akt kinase (Trencia et al., 2003), a downstream effector of Ras and PLD (Chapter III), ERK is no longer sequestered by PEA15 and can activate downstream targets. PEA15 overexpressed in epithelial cells that express constitutively active Ras is hyperphosphorylated at S116 and the overexpression potentiates Ras-mediated transformation. Interestingly, disruption of the PEA15/PLD1 complex or PLD1 enzyme inhibition with butanol or small-molecule PLD inhibitors block the PEA15 potentiation of Ras-mediated tumorigenesis suggesting that the PEA15 enhancement of PLD activity in this system drives ERK activation and AIG downstream of Ras (Sulzmaier et al., 2011). These studies strongly suggest that PLD and PtdOH transduce the signals required for Ras-induced oncogenesis, potentially through downstream activation of Raf and the MAPK pathway.

There are three members of the Raf family, A-Raf, B-Raf and Raf-1, and all activate the MAPK pathway to varying degrees with B-Raf and Raf-1 being the most potent stimulators of MEK (McCubrey et al., 2007). Raf mutations have been reported in many cancers including colorectal, ovarian, and especially melanoma, with around 60% of melanomas containing activating Raf mutations (Pollock and Melzer 2002). As such, inhibitors of mutant Raf, such as vemurafenib, are highly efficacious in treating malignant

melanoma (Bollag et al., 2012). The activation of Raf kinases is complex and generally requires membrane recruitment and subsequent phosphorylation at a number of activating residues (Yan et al., 1998). Raf contains a Ras binding domain that allows requisite membrane recruitment through interaction with activated Ras. Raf-1 can also translocate to membranes by directly interacting with PtdOH (Ghosh and Strum, 1996). Through the use of butanol and catalytically inactive PLD mutants, investigators determined that PLD-derived PtdOH bound and recruited Raf-1 kinase to facilitate ERK activation, but the interaction did not influence Raf-1 activity directly (Rizzo et al., 1999). The PtdOH binding domain was narrowed to a region of 35 amino acids and mutation of specific arginine residues abolished PtdOH binding to Raf-1. All three Raf family members contain this PtdOH binding domain. (Ghosh, 2003; Ghosh and Strum, 1996). Although the canonical Raf activation sequence involves membrane recruitment by Ras, other studies have suggested that PtdOH mediates the translocation event and Ras somehow mediates subsequent Ras activation. Evidence for this claim comes from observations that mutation of the PtdOH binding site of Raf-1 completely prevents membrane binding whereas expression of a dominant negative Ras construct inhibits Raf-1 activation but not translocation (Rizzo et al., 2000). The idea that PtdOH mediates Raf-1 translocation and Ras mediates Raf-1 activation is partially substantiated by later studies showing that Raf-1 binding to liposomes containing PtdOH is not further enhanced by Ras. However, Ras binding to the same liposomes was significantly enhanced in the presence of Raf-1, suggesting that Ras binds lipid-bound Raf-1 (Hekman et al., 2002). Therefore, PtdOH appears to directly recruit Raf-1 to membranes and possibly facilitate activation by Ras.

In addition to directly regulating Raf translocation to the membrane, PLD may indirectly affect ERK activation by regulating receptor endocytosis. Like RTKs, agonist binding to many GPCRs leads to a rapid increase in ERK activation and many of the

same components that mediate RTK activation of ERK also mediate GPCR activation of ERK. However, inhibitors of endocytosis are known to also inhibit GPCR-stimulated ERK activation (Daaka et al., 1998; Luttrell et al., 1997). Arrestins are proteins that mediate GPCR internalization and serve as scaffolds to nucleate components of the MAPK pathways (Shenoy et al., 2006). PLD activity has been shown to regulate receptor endocytosis for a variety of cell surface receptors including RTKs, such as EGFR (Shen et al., 2001), and GPCRs such as the μ-opioid receptor (Koch et al., 2003) and angiotensin II receptor (Du et al., 2004). The cone-shaped PtdOH participates in membrane fission events such as endocytosis (Barr and Shorter, 2000). However, a recent study suggested that PLD regulates receptor endocytosis independently of PtdOH. The small GTPase dynamin is a critical participant in endocytosis and functions by circling the neck of an invaginated membrane and pinching off the vesicle by constricting the neck in a manner that requires GTP hydrolysis (Ferguson and Pietro De Camilli, 2012). PLD1 and PLD2 stimulate GTPase activity of dynamin through a PtdOH-independent GAP activity in the PLD-PX domain. By using EGFR as an example, mutation of the catalytic GAP residue in the PLD-PX domain or overexpression of the PX domain fragment decreased and increased EGFR endocytosis, respectively (Lee et al., 2006). Therefore, in addition to PtdOH regulation of Ras and Raf activation, PLD may regulate MAPK signaling through GEF and GAP activities for Ras and dynamin, respectively.

Mammalian target of rapamycin

The mammalian, target of rapamycin (mTOR) is a large serine/threonine protein kinase that serves as a sensor of cellular homeostasis and responds to amino acids, stress, oxygen, energy levels, and growth factors and in turn regulates cell growth, proliferation, survival, and metabolism by phosphorylating a plethora of downstream

targets (Laplante and Sabatini, 2012). The activation pathways for mTOR are complex and depend on many factors, but a canonical activation pathway involves growth-factor receptor activation of PI3K, which activates Akt kinase through a PIP_3-dependent mechanism. Akt then activates mTOR by phosphorylating and inhibiting an upstream inhibitor of mTOR, tuberous sclerosis protein 2 (Manning et al., 2002; Shaw and Cantley, 2006). In addition to growth factor receptor mutations (Libermann et al., 1985), PI3K activity is upregulated in many cancers through inactivation of phosphatase and tensin homologue (PTEN), the lipid phosphatase that hydrolyzes PIP_3 (Haas-Kogan et al., 1998), and also though activating mutations in the PI3K catalytic domains (Engelman et al., 2006). Thus, cancers that have elevated PI3K activity frequently have elevated mTOR activity.

Growth factor activation of mTOR results in protein translation through several known substrates including p70 S6 kinase 1 (p70S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). mTOR phosphorylates p70S6K1 which in turn phosphorylates and activates the S6 ribosomal protein (Chung et al., 1992). 4E-BP1 interacts with the eukaryotic translation initiation factor 4E and inhibits translation until mTOR phosphorylation relieves the repressive function of 4E-BP1 on protein translation (Richter and Sonenberg, 2005). The bacterial macrolide rapamycin binds FKBP12 (FK506 binding protein 12) and the rapamycin-FKBP12 complex binds and inhibits mTOR (Koltin et al., 1991; Sabatini et al., 1994) at the FKBP12-rapamycin binding (FRB) domain (Choi et al., 1996). PtdOH was shown to bind R2109 in the FRB domain and an NMR structure of FRB-bound PtdOH suggested that binding of PtdOH to mTOR competes with rapamycin binding (Fang et al., 2001; Veverka et al., 2007). Some have suggested that PtdOH stabilizes mTOR complexes and high levels of PtdOH may outcompete rapamycin and explain why some systems resist rapamycin-induced cell death (Chen et al., 2003; Toschi et al., 2009). Consistent with the importance of PtdOH

in mTOR activation, the three PtdOH-generating enzymes, PLD (Fang et al., 2001), DAG kinase (Ávila-Flores, 2005), and lysophosphatidic acid acyl transferase (Tang et al., 2006) have all been shown to regulate mTOR activity. Although the literature is somewhat contradictory, both PLD1 (Fang et al., 2003; Sun et al., 2008) and PLD2 (Ha et al., 2006) have been reported as regulators of mTOR. However, small-molecule PLD inhibitors do not inhibit mTOR activity in all cells (see Chapter III), which calls into question some of the previous studies, which relied on butanol as a PLD inhibitor.

Although PtdOH can regulate p70S6K1 activity through mTOR, recent studies have demonstrated that PtdOH also directly binds and activates p70S6K1 independently of mTOR, as measured by recombinant p70S6K1 binding to PtdOH in ELISA and lipid-sedimentation based assays (Lehman et al., 2007). In order to demonstrate direct activation of p70S6K1, the authors treated cells with rapamycin to inhibit mTOR and then with PtdOH before IP of p70S6K1 and measurement of activity. Although they measured increased p70S6K1 activity after PtdOH treatment, they did not address the potential displacement of rapamycin from mTOR by PtdOH, thus calling into question a direct stimulation by PtdOH. Further studies must address whether the binding of PtdOH to p70S6K1 has physiological relevance.

Evading growth suppression

Most cells enter a state of cellular quiescence upon terminal differentiation or contact with other cells within a tissue or culture. The signals that prevent cell division and replication are diverse and complex, but are largely mediated by soluble factors and cell-surface proteins that activate inhibitors of cell cycle progression through restriction points into S (DNA synthesis) or M (mitosis) phases (Fagotto and Gumbiner, 1996). As discussed in the previous section, mitogenic signals, such as those from growth factor receptors, evoke passage through cell cycle checkpoints to replicate DNA, undergo

mitosis, and ultimately divide. In order to establish a transformed primary cell line in culture, at least two cooperating oncogenes or tumor suppressors must be mutated. These include, for example, mutations allowing constitutive mitogenic signaling, mutations that allow cells to overcome restrictions on cell cycle checkpoints, or mutations that allow cells to overcome programmed cell death (Land et al., 1983). As such, excessive proliferative signaling alone often leads to cell cycle arrest as a normal cellular response to preventing neoplastic growth. Overexpression of Raf (Woods et al., 1997) and oncogenic Ras (Serrano et al., 1997) proteins in non-immortalized, primary cells leads to cell cycle arrest. Likewise, oncogenic Ras, which does not normally transform primary cells, transforms cells with the introduction of a protein that sequesters and inactivates cell cycle checkpoint proteins (Hahn et al., 1999; Serrano et al., 1997). While PLD overexpression alone does not appear to be transforming, PLD overexpression cooperates with overexpressed proto-oncogenes such as c-Src or EGFR in rat fibroblasts (Joseph et al., 2001), suggesting that PLD might also function at the level of cell cycle progression.

The transcription factor p53 is one of the most important tumor suppressors involved in cell cycle control and over 50% of human tumors contain inactivating mutations in the p53 gene (Hollstein et al., 1994). As cells pass through the cell cycle, DNA integrity is constantly monitored. Cellular stressors such as DNA damage can activate a number of proteins including ataxia telangiectasia mutated (ATM), a well-known activator of p53 (Xu and Baltimore, 1996). Once activated, p53 either initiates a series of events triggering a cell-cycle arrest or events triggering programmed cell death. By enforcing a G1 checkpoint, p53 prevents entry into the S, phase allowing time for DNA repair to proceed. If DNA damage is sufficient, p53 can initiate programmed cell death to prevent replication and passage of damaged genes (Evan and Littlewood, 1998). p53 enforces a cell cycle arrest by upregulating a number of genes that prevent

cell cycle progression. Although there are many, the p21 cyclin-dependent kinase inhibitor (p21Cip1) is one of the best-characterized p53 targets (el-Deiry et al., 1993). p21Cip1 binds to cyclin dependent kinases and blocks their activity to enforce a G1 cell cycle arrest. p53 can initiate apoptosis by upregulating pro-apoptotic genes such as Bax (Miyashita and Reed, 1995) and death receptors (Owen-Schaub et al., 1995), and p53 can stimulate apoptosis independently of its transcriptional activity by directly activating the mitochondrial apoptotic machinery (Chipuk et al., 2004). Therefore, inactivation or suppression of p53 is often times a key step in neoplastic growth and PLD has been linked to the p53 pathway in several studies.

In normal proliferating cells, DNA damaging agents cause apoptosis through a mechanism that involves increased expression of p53. In rat fibroblasts and MDA-MB-231 breast cancer cells, overexpression of PLD1 results in decreased p53 levels and decreased apoptosis following treatment with DNA damaging agents, suggesting that PLD activity promotes p53 degradation (Hui et al., 2006; 2004). p53 levels are usually low as the protein is continuously degraded via a mechanism that involves ubiquitination and proteasomal degradation. The E3 ubiquitin-protein ligase MDM2 functions as a negative regulator of p53 by targeting the protein for proteasomal degradation (Moll and Petrenko, 2003). Overexpression of PLD correlates with increased levels of MDM2 in rat (Hui et al., 2004) and human (Hui et al., 2006) cells, potentially explaining the mechanism by which PLD decreases p53 levels. Furthermore, the possibility that PLD activity promotes p53 degradation was later supported in etoposide-treated HCT116 colorectal cancer cells when siRNA knockdown of PLD1 or treatment of cells with exogenous PtdOH increased and decreased p53 levels, respectively (Jang et al., 2008a). Additionally, overexpression of PLD1 and PLD2 inhibits etoposide-induced increase in p53 expression (Hui et al., 2004; Kwun et al., 2003). Similar results were observed in fibroblasts with high-intensity Raf signaling, normally prone to cell cycle

arrest (Woods et al., 1997). Overexpression of either PLD1 or PLD2 in these cells led to a dramatic decrease in p21Cip1 (Joseph et al., 2002). In one study, PLD2 controlled p21Cip1 expression independently of p53, raising the possibility that the PLD1 may regulate p21Cip1 levels through p53 while PLD2 may regulate p21Cip1 levels through other transcription factors such as Sp1 (Kwun et al., 2003). The molecular mechanism by which PLD regulates p53 and p21Cip1 levels largely remains to be determined. However, MAPK pathways are known to regulate p53 (Wu, 2004) and inhibitors of ERK partially negate the effects of PLD overexpression on p53 degradation (Hui et al., 2004), potentially implicating a PLD-MAPK pathway.

The retinoblastoma protein (pRB) is another frequently mutated tumor suppressor protein that functions as cell cycle restriction point guardian to allow passage from the G1 gap phase into S phase. Accordingly, loss of pRB allows unrestricted passage from G1 to S phase. pRB normally binds E2F transcription factors that, when liberated from pRB, upregulate genes necessary for G1 to S phase transition (Dyson, 1998). The regulation of pRB is complex, but multiple phosphorylation events determine whether pRB restricts S phase entry. Before cells reach the G1 restriction point, pRB is hypophosphorylated and levels of phosphorylated pRB increase during the last few hours of the G1 phase. Hyperphosphorylated pRB then allows passage through the G1 checkpoint (Weinberg, 1995) and hyperphosphorylated pRB is associated with cell cycle progression. Cyclins of the D class regulate pRB activity (Ewen et al., 1993; Kato et al., 1993) by activating cyclin-dependent kinases, which phosphorylate and activate pRB (Tamrakar et al., 2000). Although current literature does not directly implicate PLD in pRB activation, several putative links exist. First, when PLD1 or PLD2 are overexpressed in mouse fibroblasts, Cyclin D3 levels increase and a greater population of cells enter S phase relative to cells that do not overexpress PLD (Min, 2001). Additionally, p21Cip1 is a potent inhibitor of cyclin dependent kinases that phosphorylate

pRB (Harper et al., 1993), offering another mechanism by which PLD may regulate pRB activation since PLD activity is negatively correlated with p21Cip1 expression. Finally, protein phosphatase 1 (PP1), a serine/threonine phosphatase, dephosphorylates pRB and can inhibit the G1 to S phase transition (Rubin et al., 1998). PP1 is a ubiquitously expressed phosphatase in all eukaryotic cells with a broad spectrum of functions including cell division, apoptosis, protein synthesis, metabolism, cytoskeletal reorganization, and regulation of membrane receptors (Shi, 2009). PP1 is a holoenzyme consisting of catalytic and regulatory subunits that confer phosphatase specificity. PP1 activity is regulated by inhibitory proteins *in vivo* (Cohen, 2002) and PtdOH was shown to inhibit the catalytic subunit of PP1γ with an IC_{50} value of 15nM (Jones and Hannun, 2002). The PtdOH binding site was mapped to a small stretch of amino acids between 274 and 299 and mutation of serine 292 reduced PtdOH binding by about 50% (Jones et al., 2013). Theoretically, PLD activity could inhibit PP1 leading to hyperphosphorylated pRB and G1 cell cycle arrest since PP1-induced hypophosphorylation of pRB, is associated with a G1 arrest (Kwon et al., 1997) and conditions that favor pRB phosphorylation favor cell proliferation (Cobrinik et al., 1992).

Resisting cell death

Apoptosis

Along the journey to malignancy, cancer cells must overcome intrinsic programmed cell death mechanisms that exist to destroy genetically unstable or cells undergoing deregulated, autonomous proliferation. DNA damaging agents, death receptor stimulation, inhibition of oncogenic kinases, and nutrient deprivation are established stimuli of apoptotic cell death (Kelly and Strasser, 2011). As cells proliferate within a solid tumor, the nutrient supply temporarily decreases until cells can stimulate angiogenesis and restore blood flow via neovascularization. In cell culture,

viability is normally compromised following serum, or nutrient withdrawal. PLD activity is frequently upregulated in cultured cells following serum withdrawal (Zheng et al., 2006) and many cancer types require PLD and its product, PtdOH, for sustained survival under stress conditions (Foster and Xu, 2003). Several groups have thus proposed the idea that PLD and PtdOH provide some sort of survival signal to prevent programmed cell death. For example, when serum is withdrawn in rat fibroblasts overexpressing the proto-oncogene c-Src, cells undergo apoptosis unless PLD1 or PLD2 is co-overexpressed (Zhong et al., 2003). Interestingly, the same rat fibroblast line transformed with oncogenic v-Src has elevated basal PLD activity and does not undergo apoptosis following serum withdrawal, illustrating a correlation with PLD activity and cell survival. Established cancer cell lines also require PLD activity for survival in the serum withdrawal paradigm. For example, when PLD activity is inhibited in T24 bladder, Calu-1 lung, 786-O renal, HCT116 colorectal and MDA-MB-231 breast cancer cells following serum withdrawal, the established cancer lines undergo apoptosis (Kang et al., 2008; Shi et al., 2007; Toschi et al., 2008; Zhong et al., 2003). These studies utilized butanol, DN-PLD, and PLD siRNA to inhibit PLD activity and expression. The importance of PLD in the serum-withdrawal survival pathway has since been corroborated obtained using small-molecule PLD inhibitors in MDA-MA-231 cells (Lavieri et al., 2010).

Although the molecular mechanisms by which PLD protects against apoptotic cell death are not fully understood, several mechanisms have been proposed. The first mechanism involves upregulation of anti-apoptotic proteins. B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein that prevents apoptosis by binding pro-apoptotic proteins such as Bax and Bak (Cheng et al., 2001; Tsujimoto et al., 1984). Not surprisingly, overexpression of Bcl-2 is associated with apoptosis resistance in many cancers (Adams and Cory, 2007). Several cell types increase Bcl-2 expression as a function of PLD activity. PLD overexpression and exogenous PtdOH treatment in HeLa cells increases

Bcl-2 transcription (Choi and Han, 2012). H19-7 rat embryonic hippocampal cells increase Bcl-2 expression when stimulated by FGF. DN-PLD and PLD siRNA inhibit the FGF-induced increase in Bcl-2, suggesting PLD activity is required for Bcl-2 expression (Yoon et al., 2012). Both studies demonstrate that Bcl-2 transcription increases through the STAT3 transcription factor in a mechanism that requires MAPK activity. Therefore, Bcl-2 expression by PLD may be a byproduct of PLD-induced MAPK activation.

Another mechanism by which PLD may protect against apoptosis involves the regulation of mTOR. Several cell types undergo apoptosis when treated with the mTOR inhibitor rapamycin (Woltman et al., 2001). As mentioned previously, PtdOH is believed to bind and stimulate mTOR directly in a manner that competes with rapamycin binding (Chen et al., 2003). Along these lines, the pro-survival transcription factor c-Myc (Dang, 2012) is translated downstream of mTOR (Gera et al., 2004). Inhibition of PLD using butanol or DN-PLD is associated with a decrease in c-Myc protein in MCF7 cells and it has been suggested that PLD promotes c-Myc expression through mTOR activation (Rodrik et al., 2005). In some circumstances, c-Myc overexpression can force the cell to initiate apoptosis in a manner that requires the early growth response protein 1 (Egr1) transcription factor (Boone et al., 2011; Sherr, 2001). Egr1 is a tumor suppressor involved in differentiation, proliferation, and apoptosis (Boyle et al., 2009; Yu et al., 2007) and enhances expression of PTEN (Virolle et al., 2001). Apoptotic stimuli increase Egr1 expression in NIH3T3 and C6 glioma cells. However, PLD overexpression or treatment with exogenous PtdOH prevents Egr1 induction and subsequent apoptosis (Kim, 2006). These results suggest that PLD may regulate apoptosis by regulating expression of pro- and anti-apoptotic proteins, potentially through MAPK and mTOR pathways, although determination of the exact pathways should be the subject of future investigation.

Apoptosis initiation is a stepwise process involving mitochondrial permeabilization, cytochrome *c* release and activation of several caspase proteases

(Parrish et al., 2013). As such, treatment of cells with apoptotic stimuli such as small-molecule PLD inhibitors is associated with an increase in caspase activity (Lavieri et al., 2010). PLD1 and PLD2 are targets for activated caspases and are cleaved at several sites. PLD1 is cleaved at D545 and PLD2 is cleaved at D13, D16, and D28 (Jang et al., 2008b; Riebeling et al., 2008). Caspase cleavage of PLD1, but not PLD2, disrupts catalytic activity. Interestingly, the cleavage product of PLD1 inhibits uncleaved PLD1. Overexpression of this fragment inhibits endogenous PLD activity and renders cells more susceptible to apoptosis-inducing agents (Jang et al., 2008a). Likewise, expression of a caspase-resistant mutant, D545A, renders cells resistant to apoptotic stimuli (Jang et al., 2008a). These studies suggest that caspase cleavage of PLD1 leads to generation of protein fragments that enable apoptosis by inhibiting PLD activity. These studies link the intrinsic apoptotic machinery to PLD activity and further underscore the importance of PLD as a survival factor.

Autophagy

Eukaryotic cells encounter a variety of environmental stressors ranging from nutrient and growth factor deprivation to chemical stressors and infectious agents. Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process whereby cytoplasmic constituents are enveloped in double-membrane vesicles called autophagosomes and delivered to lysosomes for degradation and nutrient recycling in times of stress (Yang and Klionsky, 2010). Autophagy is the only mechanism to degrade large bulky structures, such as organelles, and serves a housekeeping function, under non-stress conditions, to clear the cell of damaged and potentially toxic components (Rabinowitz and White, 2010). In addition to providing recycled nutrients, such as amino acids and nucleic acids, autophagy is critical for immune function (Puleston and Simon, 2013) and preventing neurodegenerative diseases such as

Huntington's and Parkinson's disease by clearing toxic protein aggregates (Sarkar et al., 2007; Wang and Mandelkow, 2012). The relationship between autophagy and cancer is more complex and autophagy has been shown to serve tumor suppressing and tumor promoting roles. Mice with genetic deletions for essential autophagy genes have increased incidences of cancer, most likely due to increased genotoxic stress following buildup of damaged organelles (Edinger and Thompson, 2003). On the other hand, autophagy is required for the metabolic shift towards aerobic glycolysis in Ras-transformed fibroblasts (Kim et al., 2011b) and autophagy inhibitors decrease Ras-induced transformation (Kim et al., 2011c). Autophagy also supports tumor cell growth by clearing damaged organelles and toxic metabolites following chemotherapy treatment, thus pharmacological modulation of autophagy is clinically important (Rubinsztein et al., 2012).

Autophagy is a multi-stage process coordinated by distinct protein complexes. In yeast, approximately 30 autophagy-related (ATG) genes control the various autophagic stages, which include initiation, elongation, and maturation. Approximately half of these genes are conserved in mammals (Xie and Klionsky, 2007). Autophagosome initiation begins with the budding of a preautophagosomal structure (PAS) from a membrane within the cell. Although some debate exists as to the membrane of origin, autophagosomes may contain membrane components from the endoplasmic reticulum (Axe et al., 2008), mitochondria (Hailey et al., 2010), and plasma membrane (Ravikumar et al., 2010). PAS formation requires recruitment of several proteins including Vps34, Vps15, Atg14L, and beclin1 (Itakura et al., 2008). Atg14L targets the complex to PAS structures (Matsunaga et al., 2010) where beclin1 can stimulate the activity of the class III phosphatidylinositol 3-kinase Vps34 to increase local concentrations of PI(3)P (Kihara, 2001; Liang et al., 1999). Increased concentrations of PI(3)P contribute to the negative curvature of the PAS isolation membrane and recruit proteins containing

PI(3)P-binding domains, such as PX domains (Knævelsrud and Simonsen, 2012). Vps34 activity results in the formation of another complex containing Atg5, Atg12, and Atg16 to promote the elongation phase. Atg5 and Atg12 are conjugated to each other through the actions of Atg7 and Atg10, which function similarly to E1 and E2 ubiquitin-activating and ubiquitin-carrier proteins, respectively (Mizushima et al., 1998; Shintani, 1999). As the nascent autophagosomes elongates, Atg7, Atg3, and Atg5-Atg12 function as E1, E2, and E3 ubiquitin-like conjugation proteins to covalently attach PE to microtubule-associated protein/light chain 3 (LC3) to facilitate LC3 attachment to autophagosomal membranes (Hanada et al., 2007; Noda et al., 2011). Once LC3 attaches to the autophagosome, the Atg5-Atg12-Atg16 complex dissociates and LC3 assists in the final fusion of the PAS membranes into an autophagosome containing the engulfed cytoplasmic constituents (Nakatogawa et al., 2007). As autophagosomes mature, they fuse with endosomes to create amphisomes before ultimately fusing with lysosomes. The maturation process is less understood, but is partially mediated by the formation of beclin1-comples distinct from those formed during autophagosome initiation (Matsunaga et al., 2009; Zhong et al., 2009) and by components of the endosomal and lysosomal membranes such as Rab7 and LAMP-2 (Eskelinen, 2005).

mTOR is a classic negative regulator of autophagy and inhibits autophagosome formation by directly inhibiting components of the autophagosome initiation complex (Chang et al., 2009; Jung et al., 2010). When nutrients are widely available, mTOR inhibits autophagosome initiation by directly regulating components of the autophagy initiation complex. The formation of the Vsp34/Beclin1 complex and the subsequent autophagosome initiation is subject to regulation by proteins such as Atg1/ULK (Unc-51-like kinase), another key component of the autophagosome initiation complex (Mizushima, 2010). When nutrients are abundant, mTOR phosphorylates and binds the ULK complex, preventing autophagosome initiation (Ganley et al., 2009; Hosokawa et

al., 2009). When nutrients become limiting or cells are treated with rapamycin, mTOR activity decreases and dissociates from the ULK complex, enabling autophagosome formation. ULK is also regulated by the AMPK when AMP levels increase due to metabolic demand (Hardie, 2007; Kim et al., 2011a). In addition to mTOR and AMPK, PLD is emerging as an important, multifaceted regulator of autophagy. Vps34 directly regulates PLD1 localization and activity in response to amino acid availability or deprivation (Xu et al., 2011; Yoon et al., 2011). PLD binds to PI(3)P-containing membranes via the PX domain where it co-localizes with mTOR and promotes mTOR activity in the presence of amino acids. Amino acid deprivation decreases PLD activity (Yoon et al., 2011) and could trigger an increase in autophagosome biogenesis through decreasing mTOR activity. As such, PLD knockout mice produce fewer autophagosomes in liver slices and in embryonic fibroblasts following autophagic stimuli compared to wild-type littermates (Dall'Armi et al., 2010). Furthermore, Arf6, which is known to localize to the plasma and endosomal membranes (D'Souza-Schorey and Chavrier, 2006; Donaldson, 2003), stimulates autophagosome biogenesis from plasma membranes in a manner that requires PLD activity (Moreau et al., 2012). These observations were not directly correlated with mTOR and follow-up studies suggest PLD may independently regulate autophagy. In cells derived from polycystic kidney disease, which show upregulated mTOR activity, PLD inhibitor treatment results in a large increase in autophagosome numbers. However, rapamycin treatment of the same cells does not increase autophagosome numbers and suggests that PLD may be uncoupled from mTOR in these cells (Liu et al., 2013). Increased autophagosome numbers can result from increased biogenesis or decreased degradation, and the investigators of this study did not perform the experiments to make the distinction.

Agents that inhibit lysosomal acidification prevent autophagosome fusion and ultimate degradation. By pretreating cells with lysosomal proton pump inhibitors such as

bafilomycin A1, investigators can clamp autophagosome degradation and determine if a compound increases autophagosome biogenesis or inhibits autophagic flux (Yamamoto et al., 1998b). PLD inhibitor treatments induce a robust increase in autophagosome numbers in GBM cells, but do not increase autophagosome numbers more than bafilomycin treatment alone (chapter III). These results suggest that PLD promotes autophagosome flux and degradation. Similarly, when HeLa cells are cultured in balanced saline solutions lacking amino acids, PLD1 localizes with LC3-containing, late endosomal/lysosomal structures, but not PAS membranes suggesting that PLD promotes the later stages of autophagy as opposed to the initiation stages (Dall'Armi et al., 2010). Furthermore, PLD inhibitors increase levels of autophagy substrates in both nutrient-deprived CHO (Dall'Armi et al., 2010) and serum-deprived GBM cells (chapter III), consistent with a blockade in autophagosome degradation. Beclin1 promotes autophagosome maturation and degradation by interacting with a variety of partners throughout the process. Rubicon is a Beclin1-interacting protein that inhibits autophagosome maturation by inhibiting Vps34 lipid kinase activity (Matsunaga et al., 2009; Sun et al., 2011; Zhong et al., 2009). Akt phosphorylates Beclin1 and promotes autophagic flux by preventing the interaction with Rubicon (chapter III). PLD promotes Akt activity in GBM cells and promotes autophagic flux by preventing the binding of Rubicon to Beclin1 (chapter III).

Although most of the current data is consistent with PLD promoting autophagic flux, PLD may function to promote autophagosome biogenesis in some systems. For example, the decrease in autophagosome numbers in PLD1 KO mice is consistent with a role of PLD in biogenesis and contradicts the data seen with PLD inhibitors (Dall'Armi et al., 2010; Liu et al., 2013; Chapter III). These differences may be explained by the various treatment paradigms or by differences in the cell lines or tissues under investigation. Another possibility is that the PLD protein provides a non-lipase function

that is required for autophagosome biogenesis. Additionally, data obtained from knockout animals may expose the non-lipase functions that small-molecule inhibitors may obscure. PLD may also promote autophagy through production of other lipids such as DAG. Autophagy is upregulated when pathogens such as *S. typhimurium* infect cells. PLD-derived DAG was recently shown to be required for autophagy following infection (Shahnazari et al., 2010). Since PLD was only recently discovered to modulate autophagy, future studies are needed clarify the exact mechanism of autophagy regulation by PLD.

Activating invasion and metastasis

Once a tumor reaches a certain size, nutrient availability and space become limiting factors for continued tumor growth. The solution usually involves invasion of surrounding tissues and metastasis to distant sites where nutrients and space are not initially limiting (Hanahan and Weinberg, 2000). Invasion is a multistep process involving dissolution of the extracellular matrix (ECM) and surrounding tissue by protease secretion, detachment from neighboring cells and ECM, and finally cellular migration into the surrounding tissue. Many studies have implicated PLD in the promotion of cancer cell invasion. In early studies investigating invasive properties of human small-cell lung cancer cells, a pro-invasion role for PLD was suggested following observations that exogenously added bacterial PLD or PtdOH dramatically stimulated lung cancer cell invasion *in vitro* (Imamura et al., 1993). Later studies revealed a positive correlation between PLD activity and invasive potential. Overexpression of PLD in breast, GBM, or lymphoma cells stimulates invasion (Knoepp et al., 2008; Park et al., 2009; Zheng et al., 2006) whereas expression of DN-PLD prevents invasion (Zheng et al., 2006). Similarly, small-molecule PLD inhibitors and PLD siRNA decrease breast cancer cell invasion and further implicate the importance of PLD in invasive processes (Scott et al., 2009).

Prior to leaving their tissues of origin, cancer cells must degrade ECM components and surrounding tissues to provide a path for migration and intravasation. This process is usually mediated by production and secretion of collagenases/gelatinases termed matrix-metalloproteinases (MMP) (Rao, 2003). MMP activity is the best predictor of invasiveness in some cancers, such as GBM (Wild-Bode et al., 2001) and PLD activity is highly correlated with increased MMP activity in many cancer types. Simulation of HT1080 fibrosarcoma cells with activators of PLD such as laminin (Reich et al., 1995) or PMA (Williger et al., 1999) resulted in increased MMP2 and MMP9 secretion, respectively. By using primary alcohols and exogenously added PtdOH to decrease and increase MMP secretion, respectively, the investigators established a role for PLD in MMP secretion (Reich et al., 1995; Williger et al., 1999). Since these original studies, the requirement of PLD in MMP secretion has been established for multiple cell types including melanoma (Kato, 2005), colorectal (Kang et al., 2008), GBM (Park et al., 2009) and breast (Kang et al., 2011) cancer cells. To date, a combination of primary alcohol, DN-PLDs, PLD siRNA, and small-molecule PLD inhibitors have been used to block MMP secretion form cancer cells, firmly supporting the requirement for PLD activity. The most widely accepted mechanism for PLD regulation of MMP secretion is through a transcription-dependent mechanism. A Ras-MAPK dependent pathway likely mediates MMP transcription as DN constructs or inhibitors of multiple proteins within the MAPK-pathway block MMP secretion in response to stimuli such as PDGF, EGF, and PMA. Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a transcription factor downstream of ERK (Kurland et al., 2003) and has been named as the ERK-dependent transcription factor responsible for inducing MMP2 and MMP9 transcription (Kang et al., 2008; 2011; Kato, 2005; Park et al., 2009).

Once the surrounding tissue environment has been appropriately remodeled to permit tumor cell escape, the cells must detach from their surrounding cells and ECM before beginning the process of migration. In epithelial cells, this process is usually termed the epithelial-mesenchymal transition (EMT) where cells lose polarity and cell-cell adhesion and transition into migratory mesenchymal-like cells (Hanahan and Weinberg, 2000). During EMT, cells reorganize adhesion proteins that promote interaction with the ECM. E-cadherin is a cell-surface protein that couples to the actin cytoskeleton, regulates adhesion-dependent signaling, and interacts with other cells to transmit anti-growth signals (De Craene and Berx, 2013). Downregulation of E-cadherin is one the earliest steps in EMT, usually achieved through transcriptional regulation by a variety of pathways. Growth factors such as EGF, FGF, PDGF stimulate EMT through a Ras-MAPK pathway leading to activation of transcription factors such as SLUG, which represses E-cadherin and promotes expression of EMT genes (Bolós et al., 2003; Savagner et al., 1997; Yang and Weinberg, 2008). As PLD is intimately involved in RTK-Ras-MAPK signaling, it stands to reason that PLD may also regulate EMT and loss of cell-adhesion. Indeed, a positive correlation between PLD activity and loss of E-cadherin exists in certain cells. Wounded corneal epithelial cells undergo an EMT as cells migrate at the leading edges of the wound. Treatment of these cells with exogenous PtdOH or overexpression of PLD2 caused a dramatic reduction of cell-surface E-cadherin in these cells (Mazie et al., 2006). However, butanol did not decrease EGF stimulation of ERK in this system, suggesting that PLD may regulate E-cadherin through different means. In addition to the Ras-ERK pathway, the Wnt/β-catenin pathway also promotes EMT (Sánchez-Tilló et al., 2011; Yang and Weinberg, 2008). The Wnt pathway is critical in tissue development and is frequently mutated in many cancers (Kikuchi, 2003). Recent studies have shown that PLD1 is a transcriptional product of Wnt activation and participates in a feed-forward mechanism where PLD activity is required for transcription

of a number of Wnt-responsive genes (Kang et al., 2010). Although EMT gene expression was not measured in the study, PLD may potentially regulate EMT and loss of adhesion through Wnt. Small-molecule PLD inhibitors block Wnt-mediated transformation of NIH3T3 cells and underscore the importance of PLD in Wnt-signal transduction (Kang et al., 2010). Whether or not PLD directly participates in EMT and loss of cell-cell and ECM adhesion should be the subject of future studies.

After ECM remodeling and loss of adhesion, invading cells must migrate into surrounding tissues. Migration events occur under normal conditions such as the chemotaxis of cells towards chemical stimuli during infection and development. The process of migration requires significant cytoskeletal rearrangements and PLD is an important regulator of these events. PLD activity is required for neutrophil migration towards chemotactic peptides and cytokines such as fMLP and interleukin-8 (IL-8) (Carrigan et al., 2007; Lehman et al., 2006) and for macrophage migration towards colony stimulating factor 1 (Knapek et al., 2010). Cancer cells can secrete chemoattractants to recruit immune cells into tumors where they produce MMP and other factors that promote invasion (Condeelis and Pollard, 2006). Additionally, PLD activity is required for fibroblast migration towards LPA (Pilquil et al., 2006) and endothelial cell migration towards S1P (Gorshkova et al., 2008). Like non-transformed cells, cancer cells such as MDA-MB-231 also require PLD activity for migration (Scott et al., 2009; Zheng et al., 2006). Cellular migration is a multistep process consisting of cycles of cells protrusion, attachment to the ECM, and retraction. The first step of the cycle requires polarization of the cell into leading and retracting edges with the development of protrusions such as lamellipodia or filopodia at the leading edges. These protrusions must then be stabilized by formation of focal adhesions to the surrounding matrix, and finally, the trailing edge of the cell must detach and contract in order to move

the cell forward (Ananthakrishnan and Ehrlicher, 2007; Manneville, 2004). PLD and PtdOH have been linked to each step of the process.

PLD enzymes, especially PLD2, are frequently localized to the leading edge of motile cells in membrane ruffles and lamellipodia, depending on the cell type (Colley et al., 1997b; Nagasaki et al., 2008; O'Luanaigh et al., 2002). PLD activity is required for leading edge formation (Santy and Casanova, 2001) and overexpression of PLD promotes leading edge characteristics (Shen et al., 2002). Actin-rich leading edge formation requires a series of cytoskeletal rearrangements coordinated by members of the Rho family such as RhoA, Cdc42, and Rac (Ridley et al., 2003) along with other proteins such as non-receptor tyrosine kinases (NRTKs) of the Src family (Kanda et al., 2007). Rac proteins appear to control lamellipodia formation while Cdc42 controls filopodia formation (Manneville, 2004). As discussed previously, the RhoA family members stimulate PLD1 activity by a direct protein-protein interaction. Several studies have proposed mechanisms where Arf6 and Rac1 converge on PLD1 to promote PtdOH production and membrane ruffling in epithelial cells and mast cells (Powner et al., 2002; Santy and Casanova, 2001). However, the PLD2 isoform is perhaps more important for cell migration and an intriguing mechanism of Rac regulation by PLD2 is emerging.

In endothelial cells, Rac activation appears to require PLD2 activity as RNAi silencing of PLD2 decreased Rac activation and cell migration in response to S1P (Gorshkova et al., 2008). The PH domain of PLD2 encodes a putative CRIB (Cdc42/Rac interactive binding) domain that directly interacts with Rac proteins, located between amino acids 255-269 (Mahankali et al., 2011; Peng et al., 2011). *In vitro*, purified PLD2 potently stimulates GDP-GTP exchange on Rac2 and silencing of PLD2 leads to decreased Rac2 activation and chemotaxis in neutrophils (Mahankali et al., 2011). PLD2 also stimulates GEF activity on Rac1, although the rate of PLD2-catalyzed GDP-GTP exchange for Rac1 is less than for Rac2 (Henkels et al., 2013b). Although these studies

describe a lipase-independent mechanism of Rac activation by PLD2, other studies suggest that PLD catalytic activity is required for Rac activation (Gorshkova et al., 2008) and several lipase-dependent mechanisms have been described. In one mechanism, PtdOH directly binds the C-terminal polybasic motif of Rac1 and promotes membrane translocation (Chae et al., 2008). In the description of the PLD2-GEF activity, inclusion of PtdOH in the reaction mixture increases GEF activity (Mahankali et al., 2011), suggesting a putative mechanism where PtdOH recruits Rac1 binding to the membrane and the combination of PtdOH and PLD2-GEF activity stimulates Rac activation. Another mechanism for PtdOH mediated Rac activation involves direct modulation of the Rac GEF, dedicator of cytokinesis 2 (DOCK2). DOCK2 contains a DOCK homology region that binds PIP_3 and controls membrane localization. Full recruitment of DOCK2 to leading edges of migrating cells was shown to require PtdOH in addition to PIP_3 and the PtdOH binding site was narrowed to a polybasic amino acid stretch in the C-terminus (Nishikimi et al., 2009). DOCK2 translocation required PtdOH generated by PLD as expression of catalytically inactive PLD, treatment with butanol, and treatment with small-molecule PLD inhibitors all reduced DOCK2 accumulation at the leading edges of neutrophils undergoing chemotaxis (Nishikimi et al., 2009). In non-activated neutrophils, Rac is sequestered by GDIs. Upon activation, Rac dissociates and allows GEFs to stimulate GDP-GTP exchange leading to membrane ruffling. *In vitro*, PtdOH and other lipids such as arachidonic acid and PIP_n's stimulate dissociation of Rac from the Rac-GDI (Chuang et al., 1993). The number of mechanisms by which PLD and PtdOH stimulate Rac activity certainly highlights Rac as an important mediator of PLD-mediated leading edge formation.

Just beneath the plasma membrane lies a region rich in cortical actin known as the cortex. During leading edge formation, the cortical cytoskeleton undergoes a series of events that requires *de novo* synthesis of F-actin filaments, which provide structure

and force to the protruding end of the cell (Small et al., 1999). A complex network of actin-binding proteins sever, cap, and nucleate actin monomers to promote formation of F-actin filaments. Cortactin is an actin-binding protein that promotes nucleation and branching of actin filaments (Ammer and Weed, 2008). Cortactin is subject to regulation by NRTKs and phosphorylated cortactin promotes polymerization of actin filaments (Kim and Wong, 1998; Wu et al., 1991). In pulmonary epithelial cells, Src phosphorylation of cortactin requires PLD activity as PLD siRNA prevented Src and cortactin redistribution to the cell periphery (Usatyuk et al., 2009). In addition to Src, PLD has been shown to regulate the activity of Fer. The NRTK Fer is a cytosolic protein implicated in cell adhesion and cell migration (Greer, 2002). Cortactin is the best-characterized substrate of Fer and this phosphorylation is critical for fibroblast migration (Sangrar et al., 2007). Recently, PtdOH was shown to bind and activate Fer by binding to R417 (Itoh et al., 2009). Activation of Fer was inhibited by butanol but not DGK inhibitors suggesting that PLD-produced PtdOH was critical for Fer activation. Migration of rat kidney epithelial cells was inhibited by butanol or by genetic knockdown of PLD1/PLD2. Knockdown of Fer in addition to PLD1/PLD2 did not result in further reduction of migration suggesting PLD is a critical upstream regulator of Fer and cortactin phosphorylation (Itoh et al., 2009). Fes is NRTK that shares structural similarity to Fer and is also implicated in cell migration (Kanda et al., 2007). PLD2 interacts with Fes and overexpression of PLD2 increases Fes activity (Di Fulvio et al., 2012). Treatment of cells with exogenous PtdOH stimulates Fes activity, possibly through a direct interaction, based on sequence homology to Fer kinase (Ye et al., 2013). In addition to NRTKs, other upstream kinases such as PAK and MAPKs can activate cortactin (Campbell et al., 1999; Webb et al., 2006).

The PAKs are serine/threonine protein kinases that are activated by the small G-proteins Cdc42 and Rac. PAKs mediate many intracellular functions including

cytoskeleton rearrangement (Sells and Chernoff, 1997), stress signaling through the p38/Jun N-terminal kinase (JNK) pathways (Zhang et al., 1995), and regulation of NADPH oxidase activity by direct phosphorylation of p47phox (Knaus et al., 1995). Although the PAKs were originally identified based on the ability of Cdc42/Rac to stimulate autophosphorylation and kinase activity, sphingosine and PtdOH also potently stimulate PAK1 *in vitro* (Bokoch et al., 1998). The activation of PAK1 by Cdc42/Rac and sphingosine or PtdOH is not additive or synergistic, suggesting a common binding site on PAK1. *In vivo*, PAK1 activity is inhibited following butanol treatment, which suggests PLD-derived PtdOH regulates PAK1 activity (Chae et al., 2008). Along with Src, Fer, and MAPK, PAK1 provides another potential downstream effector of PLD capable of activating cortactin.

One of the ways in which cortactin promotes actin nucleation is to bind and activate the actin-related protein complex 2/3 (Arp2/3), which binds to a preexisting actin filament and nucleates branching of a new filament (Ammer and Weed, 2008; Weed et al., 2000). The The Wiskott-Aldrich syndrome protein (WASp) family of proteins cooperates with cortactin to activate Arf2/3 (Martinez-Quiles et al., 2004; Mizutani et al., 2002). As mentioned previously, PLD2 forms a complex with the Grb2 adaptor protein (Di Fulvio et al., 2006). WASp forms a complex with Grb2 and PLD2 and this protein complex promotes the formation of phagocytic cups in macrophages, a process requiring similar actin rearrangements to lamellipodia formation (Kantonen et al., 2011). When the Grb2 site on PLD2 is mutated, the complex fails to form properly and phagocytosis is decreased. Although a direct relationship between the PLD2-Grb2-WASp complex and cancer cell migration remains to be established, PLD is poised as a central regulator of several proteins that cooperate to promote cortical actin poymerization.

As cell protrusions extend forward, focal adhesions are formed that serve as mechanical anchors to the ECM and provide contractile forces that propel the cell forward. These adhesions are mediated in large part by the integrin family of proteins that bind ECM components and relay information about the cellular environment to intracellular components (Hood and Cheresh, 2002). Integrins attach to the cytoskeleton through a complex set of adaptor proteins including vinculin (Geiger, 1979) and talin (Burridge and Connell, 1983) and induce F-actin polymerization. Integrins are known to stimulate PLD and the integrin-induced increase in F-actin requires PLD activity. As such, butanol treatment or knockdown of PLD is associated with defects in the ability to form adhesions (Aguirre Ghiso et al., 1997; Iyer et al., 2006). The speed of migration is largely controlled by the speed at which cells form and detach from these adhesions (Lauffenburger and Horwitz, 1996) and PLD regulates migration speed in wound healing assays (Mazie et al., 2006). PLD activity is associated with an increase in vinculin levels (Mazie et al., 2006) and also activation of focal adhesion kinase (FAK), a key regulator of focal adhesion turnover (Knoepp et al., 2008; Ilić et al., 1995). Mechanistically, PLD can regulate adhesion dynamics by regulating the activity of integrin adaptor proteins. PIP_2 binding to the adaptor protein talin enables bindings to the integrin β-subunit and changes integrin conformation to a high affinity state for ECM components (Calderwood et al., 2002; García-Alvarez et al., 2003; Tadokoro et al., 2003). PLD can enhance binding of talin to integrins through regulation of PIP_2 levels by activating PIP5K. Early investigations revealed that PtdOH stimulated PIP5K activity from bovine brain membranes (Moritz et al., 1992) and later investigations revealed that only the class I PIP5Ks are stimulated by PtdOH (Jenkins et al., 1994). *In vivo* regulation of PIP5K by PtdOH has been documented as a significant decrease in PIP_2 levels in lysosomal membranes following butanol treatment (Arneson et al., 1999) and a decrease in PIP5K-

dependent actin stress fiber formation following PLD inhibition in murine fibroblasts (Pardo and Fitzpatrick, 2007). Recent studies have demonstrated reduced PIP5K activity following treatment with small-molecule PLD inhibitors (Roach et al., 2012), further underscoring the importance of PtdOH in PIP5K regulation. In mast cells, PLD inhibition was associated with decreased PIP_2 levels and reduced binding of talin to integrins (Powner et al., 2005). Thus, by regulating PIP5K and PIP_2 levels, PLD is directly able to control cell adhesion.

After protruding forward and forming focal adhesions, the retracting edge of a migrating cell must detach from the ECM and contract. This process is assisted by the formation of actin stress fibers in some systems (Kovac et al., 2013), which connect to focal adhesions (Geiger et al., 2009; Parsons et al., 2010). Stress fibers are bundles of actin filaments that provide contractile force for actively moving cells (Pellegrin and Mellor, 2007). Agents that elevate PLD activity frequently result in stress fiber formation. These include thrombin stimulation of fibroblasts (Ha and Exton, 1993), LPA stimulation of porcine aortic endothelial cells and rat fibroblasts (Cross et al., 1996; Kam and Exton, 2001), and S1P stimulation of human airway epithelial cells (Porcelli et al., 2002). In these cases, inhibition of PLD activity with butanol or DN-PLD expression prevented the agonist-mediated stress fiber formation and exogenous PtdOH stimulated stress fiber formation in the absence of agonist. Overexpression of PLD is able to induce stress fiber formation in L6 myoblasts (Komati et al., 2005) and expression of DN-PLD prevents LPA-stimulated stress fiber formation in fibroblasts (Kam and Exton, 2001). RhoA is a master regulator of stress fiber formation in many cells (Kaibuchi et al., 1999; Mackay and Hall, 1998) and RhoA-stimulated PLD activity has been implicated in stress-fiber formation. The observations that butanol and DN-PLD inhibit stress fiber formation and exogenous PtdOH stimulates stress fiber formation strongly implicate a PtdOH-dependent mechanism for stress fiber formation. Recently, however, the PX domain of

PLD2 was shown stimulate GDP-GTP exchange for RhoA directly, offering a lipase-independent mechanism for stress-fiber formation (Jeon et al., 2011). In this study, overexpression of the isolated PX domain was sufficient to trigger stress fiber formation. Therefore, PLD appears to regulate stress fiber formation through lipase dependent and independent mechanisms.

Inducing angiogenesis

Angiogenesis is the process of sprouting new blood vessels from existing vessels and is required to provide nutrients, remove metabolic waste, and promote metastasis. In this process, vascular endothelial cells are either recruited into the tumor mass or undifferentiated tumor stem cells already present undergo differentiation into epithelial-like cells to contribute to the formation of new blood vessels (Hanahan and Folkman, 1996; Wang et al., 2010b). Initiation of angiogenesis usually begins with secretion of pro-angiogenic factors that interact with cell surface receptors on endothelial cells and stimulate migration into the tumor (Colville-Nash and Willoughby, 1997; Ferrara and Alitalo, 1999). Many of these factors such as VEGF (Seymour et al., 1996), EGF, FGF, HGF (Adachi et al., 1996), and interleukin-8 (Sozzani et al., 1994) stimulate PLD activity, and PLD activity is required for VEGF induced angiogenesis (Zhang et al., 2011). In zebrafish, PLD activity is required for angiogenesis during embryonic development of vasculature (Zeng et al., 2009), further emphasizing the importance of PLD in the blood vessel devlopment.

PLD may contribute to angiogenesis in several ways. As a downstream effector of angiogenic growth factors, PLD and PtdOH may mediate growth factor signaling. Cells with elevated Raf activity demonstrate elevated VEGF expression (Akula et al., 2005). Similarly, oncogenic Ras can upregulate VEGF expression (Rak et al., 1995) and PLD may contribute to the Ras-MAPK signaling cascades in these cells. Latent forms of

VEGF can be sequestered in the extracellular matrix and become bioavailable following proteolytic processing by proteases such as MMP9 (Bergers et al., 2000; Kessenbrock et al., 2010). As such, PLD regulation of MMP9 activity may directly contribute to VEGF secretion.

Angiogenesis requires migration of endothelial cells, and PLD likely regulates the migratory processes required during angiogenesis. When mouse melanoma or lung cancer cells were implanted into WT or PLD1 KO mice, tumors in PLD1 KO mice showed a much lower density of microvascular cells (Chen et al., 2012). When VEGF-coated matrigel plugs were inserted into the same mice, endothelial cells failed to migrate to the plugs in the PLD1 KO mice, suggesting inherent defects in the migration of PLD1 KO-derived endothelial cells. Consistent with this observation, PLD1 KO mice showed impaired integrin signaling as manifested by a failure to properly adhere to ECM integrin ligands such as fibronectin, vitronectin, and collagen (Chen et al., 2012; Elvers et al., 2010). Therefore, the role of PLD in angiogenesis is most likely to mediate endothelial cell migration.

Deregulating cellular energetics

In the 1920's, the German physiologist Otto Warburg pioneered the study of cancer metabolism by characterizing fundamental differences in glucose utilization between tumors and normal tissue. These differences included avid glucose consumption and lactate production by tumor cells resulting in increased ATP production via the less efficient glycolysis pathways compared to the highly efficient mitochondrial oxidative phosphorylation pathways, even in the presence of ample oxygen (Warburg, 1956). This surprising discovery, termed the "Warburg effect," violated the established ideas of the Pasteur effect where O_2 was known to suppress glucose consumption and glycolysis (Krebs, 1972). Subsequent studies have confirmed and broadened Warburg's

initial discoveries and most cancer cells today have altered metabolic profiles consistent with the Warburg effect (Hanahan and Weinberg, 2011). When tumors outgrow the diffusion limits of nutrients and oxygen from the blood supply, a metabolic shift occurs where glycolytic gene expression increases along with increases in cell surface expression of glucose transporters. Thus, oxygen dependence is reduced compared to the more vascularized surrounding tissue. This glycolytic shift is advantageous for the cancer cell as it allows for growth and survival in the tumor microenvironment (Hsu and Sabatini, 2008). Proliferating cells must accumulate biomass such as lipids and ribosomes, and the high rate of glucose and glutamine (DeBerardinis et al., 2007) uptake provides anabolic carbons for pathways such as the pentose phosphate pathway and Krebs cycle (Vander Heiden et al., 2009). Therefore, deregulated cellular energetics is emerging as a fundamental process in tumorigenesis.

The molecular mechanisms behind the Warburg effect are not entirely characterized, but several key components have been identified including established oncogenes. For example, expression of oncogenic Ras or Myc results in an upregulation of glucose transporter activity and glycolytic gene expression (Ahuja et al., 2010; Dang and Semenza, 1999; Osthus et al., 2000; Ramanathan et al., 2005). Activation of the PI3K/Akt pathway stimulates glucose uptake and a shift toward aerobic glycolysis by regulating the activities of multiple glycolytic enzymes (Deprez et al., 1997; Elstrom et al., 2004; Majewski et al., 2004). As PLD is a signaling component in these pathways, the aerobic glycolysis induced by Ras, Myc, or PI3K may require PLD activity. As such, PLD can participate in the development of the Warburg phenotype through several different mechanisms. PLD1 is phosphorylated at S505 and activated by AMPK, a protein responsible for sensing and responding to changes in cellular energy status (Hardie, 2007; Kim et al., 2010). Glucose deprivation stimulates PLD1 activity via AMPK activation (Kim et al., 2010), suggesting PLD may be one of the early responders to

energetic perturbations as seen in a growing tumor. Glycolytic enzymes such as aldolase and GAPDH also directly regulate PLD activity, supporting the idea that PLD may serve as sensor of cellular energy homeostasis (Kim et al., 2002; 2003a). The downstream effect of AMPK-mediated PLD activation is an increase in glucose transporters at the plasma membrane (Kim et al., 2010) to facilitate glucose uptake. Likewise, several studies have shown a requirement for PLD activity for glucose transporter translocation to the plasma membrane following insulin stimulation (Bandyopadhyay et al., 2001; Huang et al., 2005; Sajan et al., 2002). PtdOH is a key component of the glucose transporter vesicular membrane and the authors demonstrated that PtdOH contributes to the late-stage fusion events. The role of PLD in exocytic events is well established in neuroendocrine cells (Vitale et al., 2001; Zeniou-Meyer et al., 2008) and similar mechanisms may be at play for glucose transporter vesicle fusion. Besides directly contributing to the biophysics of glucose transporter translocation, PLD also controls expression of glycolytic genes.

The hypoxia-inducible factors (HIF) are transcription factors that control expression of many glycolytic enzymes (Kaelin and Ratcliffe, 2008). HIF regulation is complex and involves several upstream components. Prolyl hydroxylases sense O_2, and hydroxylate residues on the alpha subunit of HIF in the presence of O_2 (Dann and Bruick, 2005). Ubiquitin is then coupled to these modified residues by the Von Hippel-Lindau (VHL) E3 ubiquitin ligase and HIF is targeted for proteasomal destruction (Schofield and Ratcliffe, 2005). Thus, loss of the VHL tumor suppressor leads to constitutively high HIF expression resulting in upregulation of glycolytic gene transcription seen in many cancers (Zhong et al., 1999). For example, 786-O renal carcinoma cells are VHL null and have constitutively high HIF levels. Butanol, PLD siRNA, and small-molecule inhibitors suppress HIF expression in these cells (Garcia et al., 2008; Toschi et al., 2008). After restoring VHL function in these cells, HIF expression

decreases and can be induced again by treatment with hypoxia mimetic compounds. PLD inhibition blocks the hypoxia mimetic-induced HIF expression and the authors attribute these results to PLD regulating HIF expression through mTOR (Toschi et al., 2008). However, as discussed in chapter II, PLD may serve an mTOR independent role as a general sensor of cellular bioenergetics.

Functions of phosphatidic acid

Phosphatidic acid is a multifunctional lipid that is generated by two other pathways besides PC hydrolysis by PLD. In the first pathway, PtdOH is synthesized *de novo* by a two-step mechanism involving the transfer of two fatty acyl chains to the *sn*-1 and *sn*-2 positions of glycerol 3-phosphate, situating PtdOH as the precursor to all other glycerophospholipids (Kennedy, 1987). In addition to *de novo* synthesis and PC hydrolysis by PLD, diacylglycerol kinase can transfer a phosphate group to DAG to generate PtdOH, although these PtdOH species are believed to be distinct in acyl composition compared to PLD derived PtdOH (reviewed in (Shulga et al., 2011). Once generated, PtdOH can mediate signaling events by indirect mechanisms through metabolism into other bioactive lipids such as LPA by phospholipase A (Aoki, 2004) and DAG by lipid phosphate phosphatase (Brindley, 2004). The most relevant functions of PtdOH for this dissertation, however, are the signaling functions mediated by PtdOH directly.

Many signaling cascades begin with the generation of bioactive lipids such as PtdOH or phosphorylated PIs that recruit or modulate enzymatic activities of downstream effectors. These transient protein-lipid interactions are mediated by families of lipid-binding domains that share some degree of sequence or structural homology (Lemmon, 2008). The C1 and C2 domains (conserved regions 1 and 2) were first identified in protein kinase C and are responsible for binding DAG and Ca^{2+}/PS,

respectively (Cho and Stahelin, 2006; Colón-González and Kazanietz, 2006). PH domains were identified as PIP_n binding domains from pleckstrin, the major substrate for PKC in platelets (Lemmon, 2008). Proteins with PH domains show some degree of specificity for PIP_n species. For example, the PH domain of Akt kinase has a high affinity for PIs phosphorylated at the D3 and D4 positions such as $PI(3,4)P_2$ and $PI(3,4,5)P_3$ but has much weaker affinity for $PI(4,5)P_2$ (Thomas et al., 2002). The PH domain of PLCδ1, on the other hand, has a high affinity for $PI(4,5,)P_2$ and this interaction is required for its catalytic activity (Ferguson et al., 1995). Monophosphorylated PI(3)P is recognized by several binding motifs including FYVE and PX domains, named after the phagocyte NADPH oxidase (Kanai et al., 2001; Kutateladze, 2006). Although most FYVE and PX domains bind PI(3)P, some have affinity for PIP_2 and PIP_3 (Song et al., 2001). Annexin domains are calcium dependent phospholipid-binding domains that are thought to predominantly bind PS, although binding to other phospholipids has been reported (Gerke et al., 2005). Besides interacting with specific lipids, other domains are known to detect or promote membrane curvature. Domains such as F-BAR, BAR, ENTH, and ANTH domains participate in functions that require membrane deformation such as endocytosis and cytokinesis (Itoh and De Camilli, 2006). The specificity and conservation of these lipid-binding domains has helped to identify proteins involved with lipid-mediated signal transduction. However, no specific domain has been identified for PtdOH binding and thus the identification of proteins modulated by PLD-generated PtdOH has been especially challenging (Stace and Ktistakis, 2006). Many of the known PtdOH binding proteins have already been described in relevant sections of this introduction. Thus, a survey of other known PtdOH-binding proteins along with the functional significance of the interactions is presented in the following section and a table summary of all PtdOH-binding proteins described in this introduction is included at the end of this section (Table 2).

Kinases

SPHK1. Sphingosine kinase phosphorylates sphingosine to produce the bioactive S1P. A family of GPCRs, known as Edg receptors, bind S1P and mediate a variety of functions including cell growth, cytoskeleton rearrangement, and vascular maturation (Spiegel and Milstien, 2002). Membrane recruitment of SPHK1 to perinuclear regions requires PtdOH (Delon et al., 2004). The translocation of SPHK1 is enhanced by overexpression of PLD or stimulation with phorbol esters and inhibited by treatment with butanol, thus suggesting that PtdOH derived from PLD regulates SPHK1 membrane localization (Delon et al., 2004).

PKCε. The epsilon isoform of PKC is a member of the calcium independent, DAG-dependent PKC family. PKCε has been implicated physiological functions such as neurite outgrowth, inflammatory or immune responses, tumorigeneis, myocardial development, and protection against ischemic damage (Akita, 2002). Membrane recruitment of PKCε requires DAG and PLD-generated PtdOH, as determined by the use of butanol (Jose Lopez-Andreo et al., 2003). In order to address the possibility that PtdOH was metabolized to DAG and did not directly influence membrane recruitment of PKCε, the authors demonstrated synergistic PKCε activation by co-stimulation with DAG and PtdOH (Jose Lopez-Andreo et al., 2003).

PKCδ. Like PKCε, the delta isoform belongs to the novel PKC family of calcium-independent, DAG-dependent PKCs. The PKCδ isoform shares partial functional redundancy to other PKC isoforms, but PKCδ mediates specific roles in apoptosis, cell cycle progression, and transcriptional regulation (Steinberg, 2004). PtdOH can stimulate PKCδ *in vitro* although the fold stimulation is much less than PS, suggesting a general requirement for anionic phospholipids (Aris et al., 1993). *In vivo* relevance of PtdOH regulation of PKCδ remains to be determined.

PKCζ. The zeta isoform of PKC is a member of the atypical family of PKCs that do not respond to calcium or DAG and appear to regulated by PIP_3 and by protein-protein interactions (Hirai and Chida, 2003; Ways et al., 1992). PKCζ is believed to participate in the MAPK cascade, NFκB transcription, the p70S6-kinase cascade, and development of cell motility (Hirai and Chida, 2003). *In vitro*, PKCζ is activated by PtdOH (Limatola et al., 1994; Nakanishi and Exton, 1992). However, mono- and polyunsaturated fatty acids, PS, and CL, also stimulate PKCζ *in vitro* (Nakanishi and Exton, 1992) and an *in vivo* regulation of PKCζ by PLD-derived PtdOH remains to be established.

PKCα. The alpha isoform of PKC belongs to the conventional family of PKCs and requires both calcium and DAG for full activation. PKCα is activated by many extracellular stimuli and plays important roles in proliferation, apoptosis, differentiation, motility, and inflammation (Nakashima, 2002). PtdOH has been shown to regulate PKCα in multiple studies. PtdOH added to PKCα-transfected COS-7 cells results in a significant upregulation of PKCα autophosphorylation (Limatola et al., 1994). Additionally, PtdOH stimulates PKCα kinase activity when added to bovine brain cytosol (Yokozeki et al., 1998). Neither study ruled out the possibility of an intermediate PtdOH-stimulated protein or metabolism of PtdOH to DAG as a potential explanation for the increase in PKCα activation following PtdOH treatment. Therefore, the exact role of PtdOH remains unclear.

Akt. A recent study demonstrated that Akt could be phosphorylated at tyrosine 176, distinct from the canonical activating phosphorylation sites of threonine 308 and serine 473. When phosphorylated at tyrosine 176, the lipid binding profile of Akt changed such that the kinase preferred to bind PtdOH, yet no functional consequence of Akt-

PtdOH binding was determined (Mahajan et al., 2010). Binding of PtdOH to Akt will be further discussed in chapter III.

Phosphatases

SHP1. The Src homology region 2 domain-containing phosphatase-1 (SHP1) is a non-receptor tyrosine phosphatase, mainly expressed in hematopoietic cells, with a wide spectrum of substrates (Soulsby and Bennett, 2009). SHP1, but not SHP2, was activated by PtdOH *in vitro* (Tomic et al., 1995). Additionally, PtdOH enhanced the association between SHP1 and EGFR and increased EGFR dephosphorylation. The high affinity PtdOH binding site on SHP1 was later mapped to the carboxy-terminal 41 amino acids (Frank et al., 1999). Although PLD was not directly shown to participate in the modulation of SHP1, PLD-generated PtdOH could act as part of a negative feedback loop involving SHP1 to terminate receptor tyrosine kinase signaling.

Lipin1β. Lipin1β is a type-1 (magnesium dependent and N-ethylmaleimide sensitive) PtdOH phosphatase that plays important roles in adipocyte differentiation by regulating triacylglycerol production and storage. Mutations in Lipin1β lead to lipodystrophy in mice (Péterfy et al., 2001). In addition to its phosphatase activity, Lipid1β translocates to the nucleus to activate a subset of peroxisome proliferator-activated receptor γ coactivator 1a (PGC-1a) target pathways including fatty acid oxidation and mitochondrial oxidative phosphorylation (Finck et al., 2006). Recently, a PtdOH binding site was mapped to a polybasic motif of Lipin1β, independent of the catalytic site. Interestingly, when these residues were mutated or when cells were treated with small-molecule PLD inhibitors, Lipin1β localized to the nucleus, suggesting that PLD and PtdOH may serve to modulate specific gene transcription (Ren et al., 2010).

Phospholipase C

PLCβ1. PI-specific PLC enzymes hydrolyze PIP_2 to generate IP_3 and DAG, which increase intracellular calcium stores and activate PKC. PtdOH directly binds purified PLCβ1 and stimulates its activity *in vitro*. The activation of PLCβ1 is unique to PtdOH as other phospholipids were unable to stimulate PIP_2 hydrolysis (Litosch, 2000). Additionally, primary butanol significantly rightward shifts muscarinic receptor-stimulated PIP_2 hydrolysis, implicating a role for PLD regulating in PLC *in vivo* (Litosch et al., 2009). Tyrosine 952 and isoleucine 955 mediate PtdOH binding, suggesting distinct modes of PLC regulation by PtdOH and $G\alpha_a$ (Litosch et al., 2009). PLCβ stimulation by PtdOH is unique to PLCβ1 as other β isoforms were unresponsive to PtdOH stimulation (Litosch, 2003).

PLCγ1. Receptor tyrosine kinase stimulation increases PIP_2 hydrolysis through the activation of PLCγ (Meisenhelder et al., 1989) and PtdOH was shown to stimulate the activity of PLCγ1 *in vitro* (Jones and Carpenter, 1993). The hypothesized mechanism for PtdOH regulation of PLCγ1 is through allosterically increasing enzyme activity rather than increasing the equilibrium dissociation constant, K_s (Jones and Carpenter, 1993).

PLCε. The epsilon isoform of PLC is activated through the actions of small G proteins such as Ras and through heterotrimeric G proteins such as $G\alpha_{12}$ and $G\alpha_{13}$ (Song et al., 2001). When purified to homogeneity, PLCε had very low basal activity that was stimulated by arachidonic acid, PtdOH, and to a lesser extent by PS and LPA (Murthy et al., 2013). Interestingly, PtdOH was a more potent stimulator of PLCε hydrolysis of PI than PIP_2 whereas arachidonic acid stimulated PLCε activity to wards both substrates equally. These results suggest that lipids may regulate substrate specificity of PLCε.

PLCδ3. The delta isoforms of PLC are activated by increased Ca^{2+}, which promotes association with PIP_2 (Rhee, 2001). Purified PLCδ3 was shown to bind PtdOH vesicles through its PH domain (Pawelczyk and Matecki, 1999) and the interaction with PtdOH increased PLCδ3 activity *in vitro.*

G protein regulatory proteins

RA-RhoGAP. As key regulators of cytoskeleton dynamics, Rho GTPases are known to control neurite development and decreased Rho activity is associated with enhanced neurite sprouting, extension, and branching (Luo, 2000). RA-RhoGAP is a Rho GAP that binds PtdOH via its PH domain (Kurooka et al., 2011). As with several other PtdOH-protein interactions, mutation of a basic residue, arginine 399, abolished PtdOH binding. The interaction of RA-RhoGAP with PtdOH stimulated GAP activity *in vitro* (Kurooka et al., 2011). Overexpression of DAG kinase increased neurite length in a manner that depended on PtdOH binding to RA-RhoGAP. Contributions of PLD-derived PtdOH were not measured in this study.

Arf GAPs. Several Arf GAPs have been identified that are modulated by PtdOH *in vitro*. Two of the earliest ArfGAPs discovered, ArfGAP1 and ArfGAP2, are both stimulated by phospholipids. ArfGAP1 activity is stimulated about 7-fold by PIP_2, PtdOH, and PS. ArfGAP2 is stimulated 2-fold by PIP_2, but not by PtdOH or PS. However, PIP_2 and PtdOH synergize to stimulate ArfGAP2 activity about 20-fold (Randazzo, 1997). ASAP1, like ArfGAP2, is weakly stimulated by PtdOH alone, but synergistic activity is observed in the presence of PtdOH and PIP_2. Like ArfGap2, PtdOH and PIP_2 synergize to activate another Arf-GAP, ASAP1. ASAP1 contains a PH domain that most likely mediates the phospholipid interactions (Brown et al., 1998b). AGAP1 is another PH domain-containing Arf-GAP that is synergistically stimulated by PIP_2 and PtdOH. Although the synergy was most robust with PtdOH, other anionic lipids such as PS and

PI also synergized with PIP_2 (Nie et al., 2002). Arf6 specific GAPs, ACAP1 and ACAP2, display similar synergy with PIP_2 and PtdOH (Jackson et al., 2000). The requirement of PIP_2 for PLD and Arf-GAP activities along with the ability of Arf to stimulate PLD presents a potential negative feedback loop whereby PLD-generated PtdOH may serve to terminate Arf signaling.

n-chimaerin. n-chimaerin was identified as a phorbol ester binding GAP for the small G protein Rac. n-chimaerin is stimulated by PtdOH and, to lesser extent, PS. Interestingly, arachidonic acid inhibits n-chimaerin GAP activity (Ahmed et al., 1993).

RGS4. The Gα subunits are regulated by a special class of GAPs known as regulators of G protein signaling (RGSs) (Ross and Wilkie, 2000). RGS proteins function to attenuate GPCR signaling by stimulating Gα GTP hydrolysis. When M1 muscarinic acetylcholine receptors were reconstituted in lipid vesicles and incubated with RGS4, vesicles containing PtdOH inhibited RGS4 GAP activity following M1-agonist stimulation. PtdOH was then determined to directly bind the first 57 amino acids of RGS4 (Ouyang et al., 2003). As discussed in chapter II, M1 stimulation activates PLD1 and thus, the inhibition of RGS4 activity by PLD-generated PtdOH might potentiate M1 signaling.

Miscellaneous proteins

NADPH oxidase. Host defense systems against invading pathogens involve phagocytosis and subsequent destruction by the generation of superoxide anions, hydrogen peroxide, and hypochlorous acid via the NADPH oxidase complex in phagocytes. This complex contains multiple membrane ($p22^{Phox}$ and $gp91^{Phox}$) and cytosolic subunits ($p47^{Phox}$, $p40^{Phox}$, $p67^{Phox}$, and Rac1/2) that are subject to regulation by PLD and PtdOH (DeLeo and Quinn, 1996). Upon engagement of cell surface receptors by pathogens, cytokines, or chemoattractants, PLD activity and PtdOH production increase (McPhail et al., 1999). PtdOH stimulates an unknown kinase that

phosphorylates the $p47^{Phox}$ and $p22^{Phox}$ subunits (McPhail et al., 1995; 1999). Later studies demonstrated that PtdOH was able to stimulate NADPH oxidase activity by directly interacting with different subunits of the complex (Palicz et al., 2001). Crystal structures of the $p47^{Phox}$ subunit revealed a high affinity binding site for PIP_2 and separate binding site for anionic phospholipids such as PtdOH. Binding of $p47^{Phox}$ to PIP_2 vesicles was synergistically increased when PtdOH was also as a vesicle component, suggesting that PtdOH increases $p47^{Phox}$ association to PIP_2 (Karathanassis et al., 2002). Recently, studies with small-molecule PLD inhibitors have confirmed the involvement of PLD in regulating NADPH oxidase (Chang et al., 2011).

Neurogranin. Neurogranin (Ng) is a protein originally identified as a PKC substrate in brain and is involved in synaptic plasticity (Baudier et al., 1991). Ng specifically binds PtdOH *in vitro* in a site that overlaps with its calmodulin-binding motif. Overexpression of PLD increased localization of Ng at dendritic spines, suggesting PLD-generated PtdOH regulates Ng localization.

Sin1. mTOR exists in two distinct complexes (mTORC1 and mTORC2) distinguished by different binding partners and unique substrate specificities. Sin1 is an essential component of the mTORC2 complex and is required for complex stability and kinase activity towards downstream effectors (Yang et al., 2006). Sin1 contains a PH domain that binds PIP_ns and PtdOH *in vitro* (Schroder et al., 2007). The relevance of Sin1-PtdOH binding is unknown, but PLD may regulate mTORC2 activity by activating or localizing Sin1.

ADP/ATP translocase. The ADP/ATP translocase (ANT) is an inner mitochondrial membrane protein that transports newly synthesized ATP from the mitochondria to the cytosol (Klingenberg, 2008). When purified from bovine heart and analyzed for bound lipids, PtdOH was tightly bound to the ANT protein (Epand et al., 2009). The physiological relevance of this interaction is unknown.

β-coatmer, Arf, N-ethylmaleimide-sensitive factor (NSF), and Kinesin. In an attempt to isolate novel PtdOH binding proteins, investigators coupled PtdOH or PIP_2 to affinity beads to isolate proteins specifically bound to PtdOH from brain cytosol. β-coatmer, Arf, NSF, and kinesin, all traffic related proteins, were identified as specifically binding PtdOH beads. Recombinant Arf and NSF were shown to directly bind PtdOH, but the physiological relevance remains unknown (Manifava et al., 2001).

Table 2. Phosphatidic acid proteins.

Protein Name	PtdOH Function	Binding Site	Reference(s)
Kinases			
Raf1	Membrane recruitment	AA 339-423	(Ghosh, 2003; Ghosh and Strum, 1996; Rizzo et al., 1999; 2000)
PKCε	Membrane recruitment	C2 domain	(Corbalan-Garcia et al., 2003; Jose Lopez-Andreo et al., 2003)
PKCζ	Activation	Unknown	(Limatola et al., 1994; Nakanishi and Exton, 1992)
PKCα	Activation	Unknown	(Nakashima, 2002; Yokozeki et al., 1998)
PKCδ	Activation	Unknown	(Aris et al., 1993)
mTOR	Activation	FRB domain - R2109	(Fang et al., 2001; Veverka et al., 2007)
p70S6K1	Activation	Unknown	(Lehman et al., 2007)
PIP5K	Activation	Unknown	(Arneson, 1999; Jenkins et al., 1994; Roach et al., 2012)
Fer	Activation	R417, R425, and H426	(Itoh et al., 2009)
GRK	Activation	Unknown	(DebBurman et al., 1995)
Akt	Membrane recruitment	PH domain	(Chapter III, Mahajan et al., 2010)
PAK1	Activation	Unknown	(Bokoch et al., 1998)
PKN	Activation	Unknown	(Khan et al., 1994; Morrice et al., 1994)
SPHK1	Membrane recruitment	C-terminus	(Jose Lopez-Andreo et al., 2003)
Phosphatases			
SHP-1	Activation	C-terminus - last 41 AA	(Frank et al., 1999; Tomic et al., 1995)
PP1cγ	Inhibition	AA 274-299	(Jones and Hannun, 2002; Jones et al., 2103)
Lipin1β	Membrane localization	Polybasic region around AA 153	(Corbalan-Garcia et al., 2003; Jose Lopez-Andreo et al., 2003)
G-protein regulators			
Rac-GDI	Inhibition	Unknown	(Chuang et al., 1993)
n-chimaerin	Activation	Unknown	(Ahmed et al., 1993)
NF1	Inhibition	Unknown	(Bollag and McCormick, 1991)
ASAP1	Activation	Unknown	(Brown et al., 1998; Randazzo, 1997)
AGAP1	Activation	Unknown	(Nie et al., 2002)
ACAP1/2	Activation	Unknown	(Jackson et al., 2000)
ArfGap1/2	Activation	Unknown	(Randazzo, 1997)
RA-RhoGAP	Activation	R399	(Kurooka et al., 2011)
RGS4	Inhibition	First 57 AA	(Ouyang et. al 2003)
SOS	Membrane recruitment	R475 and R479	(Zhao et al., 2007)
DOCK2	Membrane recruitment	C-terminus	(Nishikimi et al., 2009)
Phosphodiesterases			
PDE4D3	Activation	AA 31-59	(Grange et al., 2000; Némoz et al., 1997)
PDE4B1	Activation	Unknown	(Némoz et al., 1997)
PDE4A5	Activation	Unknown	(Bawab et al., 1997; Némoz et al., 1997)
PDE4A1	Membrane recruitment	W19 and W20	(Baillie et al., 2002)
Phospholipase C			
PLCβ1	Activation	Y952, I955	(Litosch, 2003)
PLCγ1	Activation	Unknown	(Jones and Carpenter, 1993)
PLCε	Activation	Unknown	(Murthy et al., 2013)
PLCδ3	Activation	Unknown	(Pawelczyk and Matecki, 1999)
Miscellaneous			
NADPH oxidase	Activation	R70, K55 in $p47^{Phox}$	(Karathanassis et al., 2002; McPhail et al., 1995; 1999; Palicz et al., 2001)
Neurogranin	Membrane recruitment	Unknown	(Baudier et al., 1991)
β-coatmer	Unknown	Unknown	(Manifava et al., 2001)
Arf1/6	Unknown	Unknown	(Manifava et al., 2001)
NSF	Unknown	Unknown	(Manifava et al., 2001)
Kinesin	Unknown	Unknown	(Manifava et al., 2001)
Sin1	Membrane localization	PH domain	(Schroder et al., 2007)
ANT	Unknown	Unknown	(Epand et al., 2009)
Rac1	Membrane recruitment	C-terminal polybasic motif	(Chae et al., 2008)

Research purpose

The lipid-hydrolyzing PLD enzymes respond to diverse extracellular stimuli and mediate a large number of cellular events. As such, excessive PLD activity contributes to the progression of several pathologies including cancer. The research described within this dissertation investigates the composition and functions of PLD signaling networks in order to further understand the regulation of PLD and the molecular mechanisms by which PLD promotes cancer cell survival. In chapter II, we establish model cell systems expressing either GPCRs or RTKs with affinity tagged PLD1 or PLD2 and use proteomic analyses to identify novel PLD-protein interactions. The main questions investigated in chapter II are: What are the common and unique binding partners between PLD1 and PLD2? What are the common and unique binding partners of PLD1 and PLD2 following stimulation by a GPCR or RTK? Our preliminary proteomic analysis of PLD2 complexes revealed putative interactions between PLD2 and several metabolic enzymes. Therefore, chapter II also discusses the questions of: What circumstances does PLD regulate intracellular ATP? Which types of cells require PLD activity to maintain ATP levels? What is the molecular mechanism of bioenergetic regulation by PLD? We present data to support a novel function of PLD to regulate intracellular ATP levels in certain PTEN-null GBM and breast cancer cells following serum stimulation, potentially by serving as a bioenergetic sensor.

In chapter III, we investigate a novel interaction between PLD2 and Akt kinase and determine the mechanism by which PLD2 promotes GBM cell survival by regulating the activation of Akt. The main questions addressed in chapter III are: How does PLD2 control activation of Akt kinase? How do PLD2 and Akt promote GBM cell survival? What are the molecular targets for PLD2-stimulated Akt activity? We present a mechanism by which the cell stressor of serum withdrawal stimulates PLD2 activity

leading to PtdOH-mediated membrane recruitment and activation of Akt, which promotes autophagic flux and ultimately cell survival in GBM cells. We demonstrate PLD to be a clinically relevant target in GBM as PLD inhibitors decrease Akt activation, autophagic flux, and ultimately GBM cell viability.

CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF NOVEL PLD COMPLEXES

Rationale

At the beginning of this project, the molecular mechanisms for the cellular functions of PLD were not well characterized, partially due to incomplete characterization of the PLD interactome. The majority of PLD-interacting proteins were purified and identified from extracts of tissues and cells based on their ability to stimulate or inhibit PLD activity and were limited to a relatively small number of proteins. Since their identification, proteins such as Arf, Rho, and PKC have been extensively characterized for their ability to activate PLD downstream of cell-surface receptors. Given the ubiquitous nature of PLD activation by diverse extracellular stimuli, it seems likely that additional unidentified proteins also participate in receptor-mediated PLD activation. Once activated, PLD mediates diverse cellular functions such as proliferation, migration, secretion, trafficking, protein synthesis, and cytoskeletal rearrangements depending on the stimulus and type of cell. Investigators have attempted yeast two-hybrid studies to yield insight into the molecular mechanisms for these PLD-mediated functions, but these studies have only identified a small-number of PLD interacting proteins (Koch et al., 2003; Zhang et al., 2000). To date, no studies have been published that used an unbiased systems biology approach to identify binding partners of PLD enzymes. Therefore, we utilized proteomic approaches to identify and characterize novel PLD complexes formed after various receptor stimulations and we believed this approach would allow us to answer several important questions. The first question concerns determination of proteins that are common and unique for activation of PLD1 and PLD2 following GPCR and RTK stimulation. The second question concerns identification of

PLD-effector complexes that form following unique receptor stimulations. By answering these questions, we hoped to further understand the protein components of PLD signaling networks.

Development of model systems

This project began with the goal of identifying novel regulators and effectors of the individual human PLD isoforms and to further understand the role of PLD in intracellular signaling. HEK293-TREx cells were chosen as a model system due to their low levels of endogenous PLD activity and their high transfection efficiency. The M1 muscarinic receptor, a typical GPCR, and EGFR, a typical receptor tyrosine kinase, are known to activate PLD following receptor stimulation (Fisher et al., 1991; McKenzie et al., 1992). Preliminary data indicated that the M1 receptor coupled exclusively to PLD1 whereas the EGFR coupled to both PLD1 and PLD2. In these studies, PLD1 or PLD2 was overexpressed in HEK293 cells, which stably expressed either M1 or EGFR, and PLD was stimulated using an agonist for each receptor. Expression of PLD1, but not PLD2, potentiated M1 stimulation of PLD activity (Figure 1A). On the other hand, stimulation of the EGFR resulted in activation of both PLD1 and PLD2 (Figure 1B). The specificity of receptor-PLD signaling was then confirmed genetically using small interfering RNA (siRNA). Silencing of endogenous PLD1 abolished the M1 response whereas silencing of both PLD1 and PLD2 was required to completely inhibit the EGFR response (data not shown). These model systems thus provided a unique background for studying isoform-specific PLD signaling.

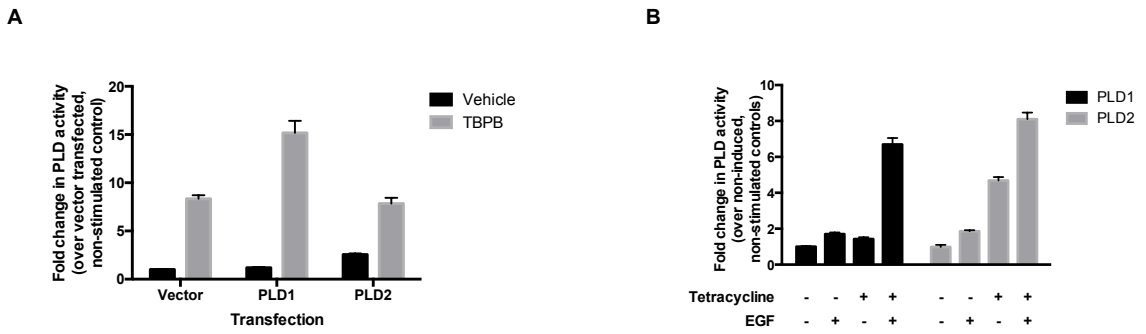

Figure 1. PLD1 and PLD2 activity following EGF or M1 receptor stimulation. **A**. HEK293-M1 stable cells were transiently transfected with the indicated human PLD isoform or vector control for 48 hours prior to assay. Cells were stimulated with 1 μM M1 agonist TBPB (Bridges et al., 2008) in the presence of *n*-butanol-d_{10} for 30 min prior to glycerophospholipid extraction and subsequent phosphatidylbutanol (PtdBuOH) quantification. **B**. HEK293-EGFR cells were stably transfected with tetracycline-inducible PLD1 or PLD2 to generate monoclonal cell lines. Cells were induced with tetracycline for PLD1a or PLD2 overexpression 72 hours prior to assay. Cells were serum starved overnight and stimulated with 100 ng/ml EGF for 15 min before measuring PLD activity as in (A). Error bars – standard error of the mean (SEM) of triplicate samples

After establishing a model cell system, we developed a method for isolating PLD complexes from agonist-stimulated cells and the general experimental scheme is presented in Figure 2. Epitope-tagged PLD constructs were expressed in either the M1 or EGFR HEK293 cells, stimulated with appropriate agonist, and isolated using immunoprecipitation or other affinity capture techniques. Several epitope tags were tested for immunoprecipitation suitability including myc, HA, FLAG, and hexahistidine. All epitope tags were fusted to the amino-terminus as any modification to the carboxy-terminus results in a catalytically inactive PLD enzyme (Liu et al., 2001). All constructs expressed at similar levels, and we used the myc-tagged PLD construct for initial proteomic analyses.

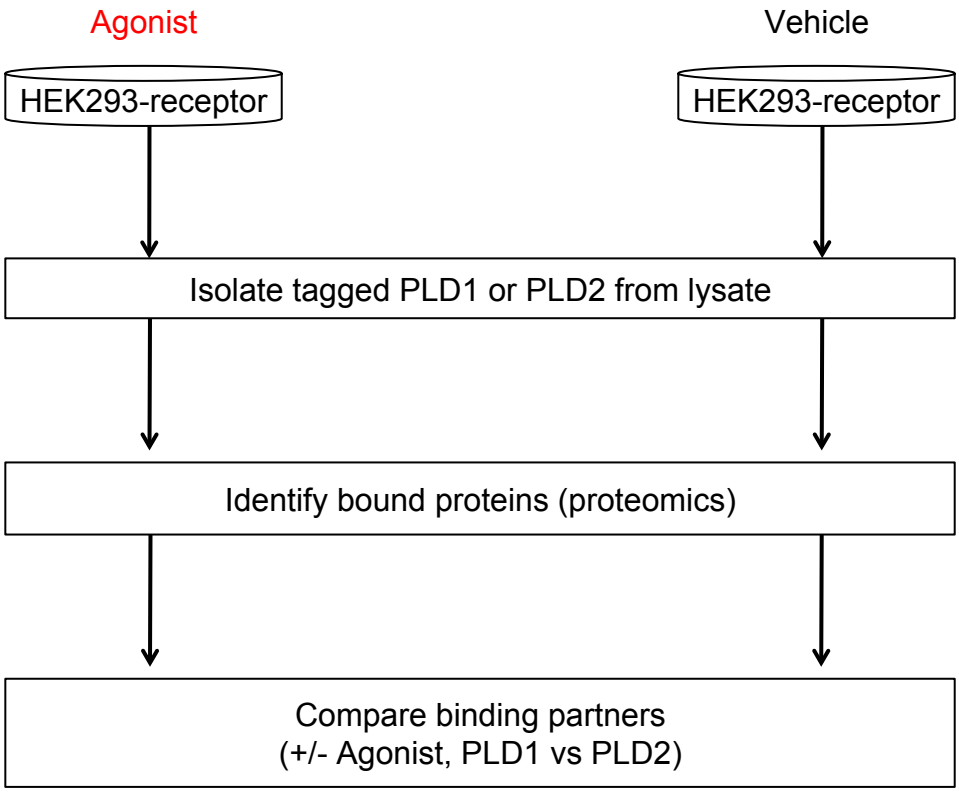

Figure 2. Experimental scheme for identifying receptor-stimulated PLD complexes. Parental HEK293-TREx cells were stably transfected to overexpress either the M1 muscarinic or EGF receptor. HEK293-EGFR or HEK293-M1 cells were then transfected with epitope tagged PLD1 or PLD2 and stimulated with the appropriate agonist. Epitope-tagged PLD was immunoprecipitated and submitted for proteomic analysis to compare binding partners in the presence and absence of agonist stimulation.

An example myc-PLD1 IP experiment can be seen in Figure 3. Several problems arose from this original approach: high levels of non-specific binding in control conditions, contamination from the heavy and light chain immunoglobulins (IgG) (55 kDa and 30 kDa bands), and low levels of expression for myc-PLD1. As such, our first proteomic analysis of M1-stimulated PLD1 complexes confirmed that the majority of proteins were present in both PLD1 and vector controls, consistent with high levels of non-specific binding and weak expression of myc-PLD1.

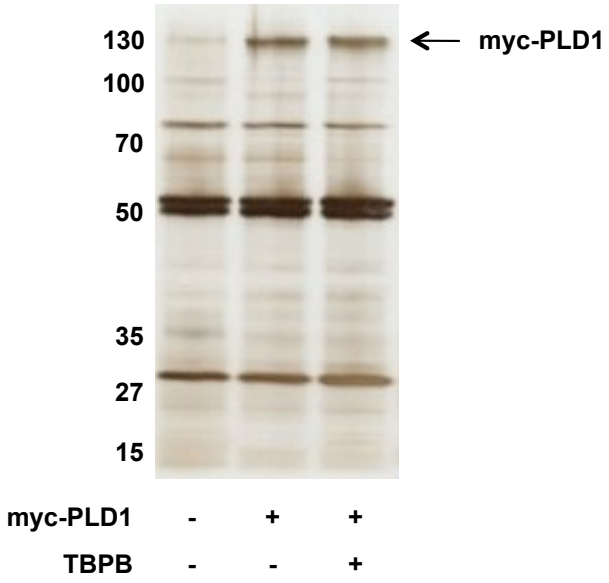

Figure 3. Immunoprecipitation of PLD1a-myc. HEK293-M1 stable cells were transfected with myc tagged PLD1a for 48 hours before stimulation with 1 μM TBPB for 5 min. Cells were lysed and PLD1 immunoprecipitated with an anti-myc antibody. Complexes were eluted in SDS loading buffer, separated on SDS-PAGE gels, and silver stained to assess PLD1-protein complex formation. Numbers indicate molecular weights of standards in kDa.

Tandem affinity purification of PLD2

In order to circumvent the problems associated with high levels of non-specific protein binding, we created new PLD constructs expressing a tandem affinity purification (TAP) tag that binds affinity resin and can be gently eluted using small molecules. The TAP procedure generally consists of two different affinity tags separated by a TEV protease cleavage site (Rigaut et al., 1999). This technique has been widely used in yeast as well as mammalian proteomics due to the drastic reduction in non-specific proteins and from the lack of heavy and light chain IgG contaminants (Knuesel et al., 2003; Rigaut et al., 1999). We chose a TAP tag consisting of the IgG binding domain of protein-A from *Staphylococcus aureus* and the strepII tag, which binds strep-tactin affinity resin, to create the ProteinA-TEV-StrepII (PtS) tag (Giannone et al., 2007). Amino-terminal, PtS-tagged PLD constructs were ligated into the pcDNA5/TO vector

backbone, which encodes tetracycline operator sequences in the promoter region that allows for transcriptional control in cells that express the tetracycline repressor protein. The HEK293-TREx line was engineered by the manufacturer to stably express the tetracycline repressor protein. We created stable cell lines for PtS-PLD1 or PtS-PLD2 in each receptor background, and determined that PtS-PLD2 expression levels were much higher than PLD1. Therefore, the remaining effort was focused on isolating PLD2 complexes.

In the TAP procedure, cell lysates are applied to IgG agarose, washed extensively, and then incubated with TEV protease overnight to liberate the strepII-tagged PLD2 from the IgG agarose. Proteolyzed strep-PLD2 is then applied to strep-tactin affinity resin, washed extensively, and eluted with biotin. Given the multiple rounds of affinity purification in the TAP procedure, final eluates should contain fewer non-specific proteins. However, the multiple rounds of affinity purification also provide more opportunities for protein loss. Therefore, we determined the capture efficiency and protein loss at each step (Figure 4). Protein expression was induced in HEK293-EGFR-PtS-PLD2 cells for 48 hours prior to cell harvest and detergent lysis. Lysate was clarified by centrifugation at 500 × *g* for 10 minutes and the supernatant was saved as the detergent soluble lysate (Figure 4A, lane 2). The insoluble pellet was boiled in 1% SDS and saved as the insoluble fraction (Figure 4A, lane 1). We noted that the TAP tag seemed to enhance PLD detergent solubilization more than any of the small epitope tags. To capture the protein-A tag, lysate was incubated with IgG-agarose for 3 hours in a Bio-Rad disposable poly-prep column. After incubation, lysate was allowed to flow through the column (Figure 4A, lane 3). Resin was washed three times with 10 ml lysis buffer and once with TEV cleavage buffer (Figure 4A lanes 6-9). Beads were resuspended in TEV cleavage buffer, transferred to 1.5 ml centrifuge tubes, and 50 units of TEV protease were added overnight. Aliquots of IgG agarose, before and after TEV

cleavage, were saved to determine proteolysis efficiency (Figure 4A lanes 4 and 5). TEV-cleaved PLD2 was then applied to strep-tactin resin (Figure 4A, lane 10) and allowed to incubate for 3 hours before centrifugation of resin and collection of non-bound material (Figure 4A, lane 11). Resin was washed three times (Figure 4A, lanes 12-14) and then PtS-PLD2 was eluted in three 500 μλ fractions in buffer containing biotin (Figure 4A, lanes 16-18). In order to determine biotin elution efficiency, an aliquot of strep-tactin resin post-elution was probed for PLD2 (Figure 4A, lane 15). Proteins were precipitated from the large wash and elution volumes using trichloroacetic acid (TCA), allowing for a quantitative measure of protein levels between fractions.

Tracking the capture efficiency and protein loss for PtS-PLD2 allowed us to draw several important conclusions. First, the majority of PtS-PLD2 is captured by the IgG and strep-tactin resins although some material remains unbound. Second, TEV proteolysis yields two PtS-PLD2 immunoreactive bands corresponding to the full-length PLD2 and PLD2 lacking the protein-A tag (Figure 4A, lanes 4 and 5). The strong presence of these two bands indicates that a large percent of PLD2 was not proteolyzed by TEV and that the cleaved product either binds non-specifically to the IgG resin or forms a dimer with the uncleaved PLD2. Finally, the final yield of PLD2 from strep-tactin is a small fraction of the PLD2 starting material as measured by immunoreactivity in the lysate before IgG-agarose incubation. Thus, although the final eluates were much less contaminated than in IP immunoprecipitation (Figure 4B), the use of the TAP procedure for large-scale protein purification would likely result in minimal protein yield. However, since mass spectrometry is a highly sensitive method of identifying even low abundance peptides, we proceeded to analyze PtS-PLD2 complexes from HEK293-EGFR cells.

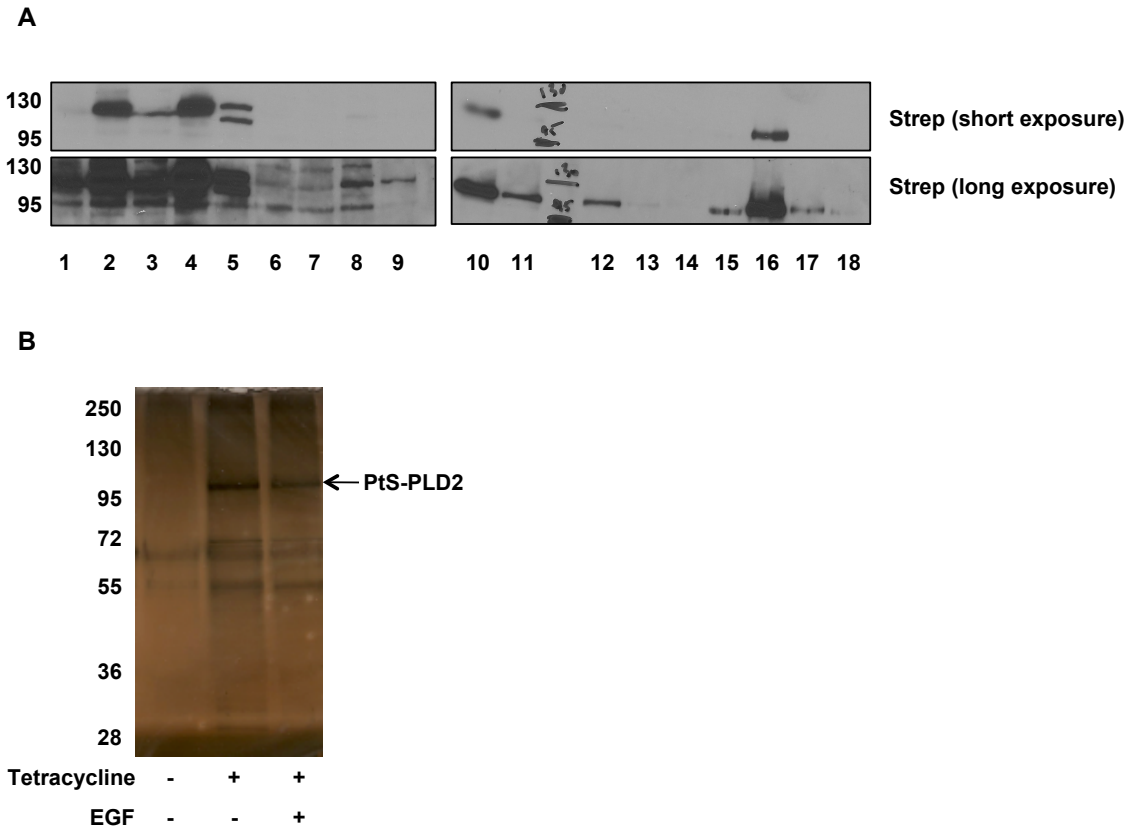

Figure 4. Determination of capture efficiency and purity of PtS-PLD2 tandem affinity purification. **A**. Fractions from PtS-PLD2 purification were immunoblotted for PLD2. Lane 1: 20 mg of insoluble pellet following cell lysis. Lane 2: 20 mg of cell lysate before incubation with IgG beads. Lane 3: Flowthrough after IgG incubation (same volume as lane 2). Lane 4: Aliquot of IgG beads before TEV cleavage (5% of total IgG volume). Lane 5: Aliquot of IgG beads after TEV cleavage (5% of total IgG volume). Lane 6: Wash 1 (25% of total wash material). Lane 7: Wash 2 (~25% of total wash material). Lane 8: Wash 3 (25% of total wash material). Lane 9: Wash 4 (25% of total wash). Lane 10: 10% of TEV-cleaved PLD2 products. Lane 11: 25% of flowthrough after strep-tactin incubation. Lane 12: Strep-tactin wash 1 (25% of total wash material). Lane 13: Strep-tactin wash 2 (25% of total wash material). Lane 14: Strep-tactin wash 3 (25% of total wash material). Lane 15: Strep-tactin resin after elution with biotin (boiled in SDS loading buffer, (33% of resin material). Lane 16: Elution 1 (50% of total elution). Lane 17: Elution 2 (50% of total elution). Lane 18: Elution 3 (50% of total elution). **B**. Silver-stained gel of final eluates from TAP procedure. HEK293-EGFR-Pts-PLD2 cells were induced with tetracycline for 48 hours before stimulation with EGF and tandem affinity purification of PLD2 complexes. Numbers indicate molecular weights of standards in kDa.

Proteomic analyses of PLD2 complexes from EGFR stimulated HEK293 cells

Our second proteomic experiment contained three conditions: non-induced HEK293-TREx-EGFR-PtS-PLD2 cells as a negative control, tetracycline-induced cells stimulated with 100 ng/ml EGF for 5 minutes, and tetracycline-induced cells with no EGF stimulation. Cells were harvested and PtS-PLD2 isolated using the full TAP procedure. Biotin eluates were pooled, precipitated with TCA, and the entire TCA precipitate run on denaturing SDS-PAGE gels, which were delivered to the Vanderbilt proteomics core. Peptides were identified after in-gel trypsin digestion and separation on a one-dimensional high-performance liquid chromatography (HPLC) column by tandem mass spectrometry. Although the list of identified interactions was small, the second proteomic analysis was much more successful than the first attempt since several proteins were identified in the tetracycline-induced samples that were not present in the non-induced samples. Briefly, these included known interacting proteins such as tubulin (Chae et al., 2005) and novel potential interactions such as the small GTPase Rab11 and various components of the ATP synthase holoenzyme. We concluded that the full TAP procedure resulted in the loss of weakly interacting proteins and most likely prevented identification of interesting PLD2 interactions. Therefore, we abandoned the full TAP procedure and isolated PLD2 complexes using strep-tactin resin alone.

By using a single affinity step to isolate PtS-PLD2, we captured greater quantities of PLD2 with visible bands that were not present in non-induced controls (Figure 5). Therefore, we repeated the proteomic analysis using the single strep-tactin affinity capture. HEK293-EGFR-PtS-PLD2 cells were induced with tetracycline and stimulated with EGF as in the second proteomic experiment. We included another set of cells transfected with an empty vector to account for any leakiness of the tetracycline-induction system. PtS-PLD2 was isolated using strep-tactin, eluted in three fractions per condition, and proteins were precipitated from pooled fractions with TCA. Proteins were

delivered to the proteomics core as a dried film to improve detection of proteins that might not be efficiently extracted from acrylamide gels. The third proteomic analysis also used a multi-dimensional HPLC approach (MudPIT; multi-dimensional proteomic identification technology) to improve resolution and identification of peptides (Washburn et al., 2001). Trypsin-digested peptide mixtures were resolved by strong cation and reversed phase chromatography before peptide fragmentation and identification by tandem mass spectrometry. Our third proteomic analysis identified many more novel PLD2 interactions compared to the previous two attempts. These PLD2-protein interactions can generally be grouped into five functional categories including metabolism, heat shock or chaperone, transport, signal transduction, and trafficking. A table of these results can be found in Table 1. Although we identified many proteins in the PLD2 samples not present, or in low abundance, in the vector control samples, we were surprised to find no unique proteins in the EGF-stimulated conditions. One explanation is that overexpressed PLD2 already has significant basal activity (Figure 1) and that EGF stimulation only yields about a two-fold activation. The proteins required to activate PLD2 following EGF treatment might constitutively bind PLD2 in this overexpression system and mask differences between basal and agonist-stimulated conditions. Another explanation is that the PLD2-protein interactions following EGF treatment might be transient or low affinity interactions and dissociate following cell lysis and protein capture. Future studies should use chemical cross-linking reagents to capture these low affinity interactions. Due to the lack of unique proteins identified in EGF-stimulated conditions, we shifted the focus to investigating a role for PLD2 in cellular bioenergetics, based on the large number of metabolic enzymes identified as potential PLD2 binding partners.

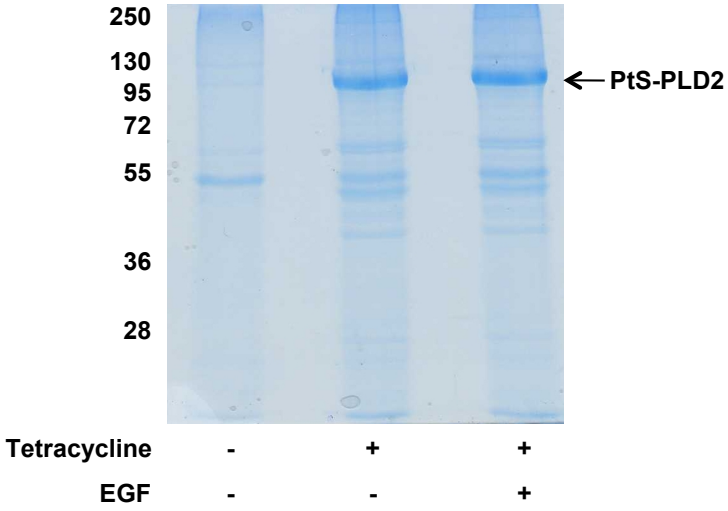

Figure 5. Strep-tactin affinity capture of PtS-PLD2 complexes. HEK293-EGFR-PtS-PLD2 cells were induced with tetracycline for 48 hours prior to EGF stimulation. PtS-PLD2 complexes were captured using strep-tactin affinity resin and eluted with biotin. Eluates were precipitated with TCA, run on SDS gels, and stained with coomassie blue.

Table 1. Proteomic analysis of PLD2 complexes.

Metabolic enzymes	Heat Shock / Chaperone	Transport	Signal Transduction	Trafficking
ATP synthase subunit α	Isoform 1 of heat shock protein HSP 90 α	ATP/ATP translocase 3	Isoform 1 of DNA-dependent protein kinase catalytic subunit	Coatomer subunit β
ATP synthase subunit β	Heat shock protein HSP 90 β	ATP/ATP translocase 2	Protein phosphatase PP1 alpha catalytic subunit	Coatomer subunit γ 2
ATP synthase subunit O	DnaJ homolog subfamily A member 1	Neutral amino acid transporter B	Protein phosphatase-2A 65 kDa regulatory subunit Aα	Adaptor-related protein complex 2
ATP synthase subunit g	DnaJ homolog subfamily A member 2	Isoform SERCA 2A or 2B of sarcoplasmic/endoplasmic reticulum Calcium ATPase 2	Protein phosphatase 2A catalytic subunit α or β	Isoform 1 of AP-3 complex subunit β1
Isoform heart or liver ATP synthase γ chain	DnaJ homolog subfamily A member 4	Isoform A-E, or K of plasma membrane Ca^{2+}-transporting ATPase 1	Guanine nucleotide binding protein subunit alpha-11	Isoform 1 or 2 of AP-2 complex subunit beta-1
Isoform 1 or 2 of ATP synthase f chain	DnaJ homolog subfamily B member 11 precursor	Transportin 1 isoform 1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 1	Vesicle-associated membrane protein 3
ATP synthase-coupling factor 6	DnaJ homolog subfamily B member 12	Isoform 1 of import inner membrane translocase subunit TIM50	Transducin beta-like 3	Vesicle-trafficking protein SEC22b
Isoform 1 of ATP synthase D chain	T-complex protein 1 subunit alpha	ATP-binding cassette subfamily B member 10	Transferrin receptor protein 1	Syntaxin-binding protein 2
ATP synthase B chain	T-complex protein 1 subunit beta	Metaxin 1	Integrin beta 1 isoform 1A precursor	Ras-related protein Rab21
CTP synthase 1	T-complex protein 1 subunit gamma	Exportin 1	Isoform 1,3, or 4 of epidermal growth factor	Ras-related protein Rab11B
L-lactate dehydrogenase B chatin	T-complex protein 1 subunit delta	Isoform 1 or 3 of Exportin 2	Insulin receptor substrate 4	Ras-related protein Rab14
Isoform 1 or 2 of L-lactate dehydrogenase A chain	T-complex protein 1 subunit zeta	Isoform 1 of Exportin 5	Isoform 1 of Basigin precursor	Ras-related protein Rab5C
NADPH dehydrogenase [ubiquinone] iron-sulfur protein 3	T-complex protein 1 subunit eta	Isoform 1 or 2 of Importin 4	GCN-like protein 1	Ras-related protein Rab2A
NADPH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	BAG family molecular chaperone regulator 2	Importin subunit beta-1	Calpain-2 catalytic subunit precursor	
Isoform alpha of alpha enolase	Isoform 1,4, or 5 of tubulin-specific chaperone D			
Phosphoenolpyruvate carboxykinase [GTP]				
Hypoxanthine-guanine phosphoribosyltransferase				
Ornithine aminotransferase				
Succinyl-CoA ligase [GDP-forming] beta-chain				
Epoxide hydrolase 1				
Fatty acid desaturase 2				
Fatty acyl-CoA reductase 1				
Lanosterol synthase				
Serine palmitoyltransferase 2				
Leucine-rich PPR motif-containing protein				

Proteins listed in this table were identified predominantly in PLD2 samples and not in vector controls. If peptides were present in the vector control samples, only peptides with a ratio greater than 4:1 PLD2:vector were included in the table of putative PLD2 interacting partners. Note, no proteins were identified that were unique to EGF stimulated conditions.

Validation of the interaction of PLD2 with ATP synthase

The most predominant hits from the second and third proteomic analyses were enzymes associated with metabolism, and especially components of the ATP synthase complex. This 500-kDa complex functions to drive synthesis of ATP from ADP and inorganic phosphate in the mitochondrial matrix. ATP synthase contains an inner mitochondrial membrane-associated component (F_O) that forms a pore through which H^+ ions can flow down their electrochemical potential gradient previously established by the electron transport chain. The energy from the flow of H^+ through the F_O component provides the energy needed to drive rotational catalysis of the F_1 catalytic component. The F_O and F_1 components each contain multiple subunits that are remarkably conserved among species ranging from plants and bacteria to humans: a, b, and c for F_O and α, β, δ, ε, and γ for F_1 (Boyer, 1997). The majority of these subunits were identified in the PLD2 proteomic screen (Table 1). Since these are highly abundant proteins that frequently present as false-positives in proteomic analyses, we validated the interaction with immunoprecipitation experiments from HEK293-TREx cells.

In the first experiment, myc-PLD2 was transfected into HEK293-TREx-EGFR cells and cells were stimulated with 100 ng/ml EGF as in the experiments for proteomic analysis. By using myc-PLD2 instead of the PtS-PLD2, we were able to exclude non-specific binding of proteins to the PtS tag. Myc-PLD2 was immunoprecipitated with a myc antibody and the complexes were probed for co-IP of endogenous ATP synthase alpha. To exclude non-specific interactions of ATP synthase with the protein-G agarose or the myc antibody, we immunoprecipitated non-specific proteins from an empty-vector transfected set of HEK293-TREx-EGFR cells. As can be seen in Figure 6A, we detected specific binding of endogenous ATP synthase alpha to myc-PLD2. Next, we performed the reverse IP experiment where we immunoprecipitated endogenous ATP synthase alpha and probed for co-IP of myc-PLD2. HEK293-TREx cells were transfected and

stimulated as in Figure 6A. To exclude non-specific binding of myc-PLD2 to protein-G or to mouse IgG, we immunoprecipitated non-specific proteins from myc-PLD2 transfected cells using an antibody of the same isotype as the ATP synthase alpha antibody. As can be seen in Figure 6B, myc-PLD2 co-immunoprecipitated specifically with ATP synthase alpha. The proteomic analysis of PLD2 identified no proteins that uniquely interacted with PLD2 following EGF stimulation, and the forward and reverse immunoprecipitation experiments with PLD2 and ATP synthase alpha confirmed the constitutive, EGF-independent interaction.

Along with the alpha subunit, we confirmed the interaction of PLD2 with the beta subunit of ATP synthase. A PLD2 construct containing the strepII tag was created to remove the bulky Protein-A tag component of the TAP tag. StrepII-PLD2 or empty vector was transfected into HEK293-TREx-EGFR cells and PLD2 complexes were isolated using strep-tactin as in the proteomics experiment. StrepII-PLD2 was eluted from resin by boiling in 2x SDS-PAGE loading buffer and complexes probed for ATP synthase beta. Although the commercial ATP synthase beta antibody was not as robust as the alpha antibody, a faintly immunoreactive band can be detected for ATP synthase beta in the strepII-PLD2 sample (Figure 6C). Together these results confirm the proteomic identification of ATP synthase as a bona fide PLD2-interacting protein.

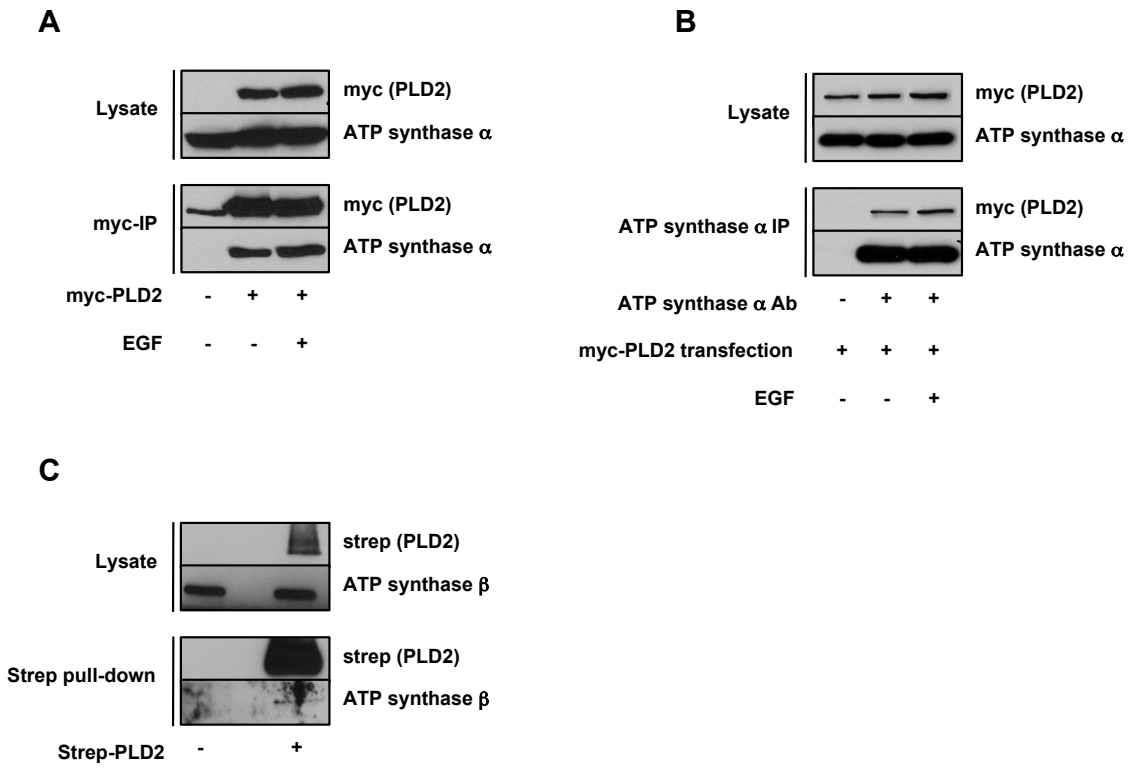

Figure 6. Validation of the PLD2 interaction with ATP synthase. **A.** myc-PLD2 or vector was transfected into HEK293-EGFR cells for 48 hours prior to stimulation with EGF. Cells were subsequently lysed and myc-PLD2 complexes were immunoprecipitated with a myc-specific antibody. Complexes were probed for co-IP of endogenous ATP synthase α. **B**. Endogenous ATP synthase α was immunoprecipitated from myc-PLD2 transfected HEK293-EGFR cells and complexes were probed for co-IP of myc-PLD2. Non-specific proteins were immunoprecipitated by an antibody of the same isotype as the ATP synthase α antibody. **C**. HEK293-EGFR-strep-PLD2 cells were induced with tetracycline for 48 hours prior to cell lysis. Strep-PLD2 complexes were captured with strep-tactin affinity resin and complexes were immunoblotted for ATP synthase β.

Validation of other proteins identified in the PLD2 proteomic analysis

Similar IP and pull-down experiments were used to validate two other protein interactions. For the first interaction, we attempted to confirm the binding of PLD2 to the ADP/ATP translocase 2 (ANT2) by immunoprecipitating PLD2 and probing for co-IP of ANT2. Unfortunately, the commercially available antibodies for ANT2 were not sensitive enough to detect co-IP of endogenous levels of protein (Figure 7). For the second interaction, we attempted to confirm the binding of PLD2 to heat shock protein 90

(HSP90). HEK293-TREx-EGFR cells were transfected with myc-PLD2 and PLD2 immunoprecipitated using a myc antibody to probe for endogenous HSP90. No interaction was detected using myc-PLD2 as bait. To test the possibility that the either the protein-A or strepII tags non-specifically interacted with HSP90, we transfected either the full-length TAP tagged PLD or the strepII tagged PLD2 into HEK293-TREx cells and isolated PLD using strep-tactin affinity resin. As can be seen in Figure 7, binding of HSP90 was only detected using the full-length TAP tagged PLD2, confirming that HSP90 interacts with the Protein-A tag and not PLD2. Since the identification and validation of the PLD2-ATP synthase interaction provided a completely novel path for interrogation of PLD function, other proteomic identifications were not validated.

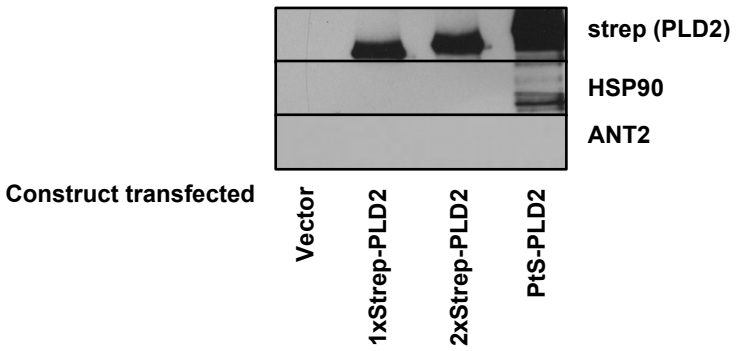

Figure 7. Validation of HSP90 and ANT2 as PLD2-interacting partners. HEK293-TREx-EGFR cells were transfected with PLD2 containing an N-terminal strepII tagor PtS tag 48 hours prior to cell lysis and capture of PLD2 using strep-tactin affinity resin. Complexes were probed for HSP90 and ANT2. Binding partners of strep-PLD2 were compared to PtS-PLD2 to control for non-specific protein binding to the PtS tag.

Regulation of cellular bioenergetics by PLD

Growth factor stimulation of intracellular ATP

The robust interaction between PLD2 and ATP synthase prompted us to investigate a potential role for PLD in the regulation of bioenergetics. The previously discussed IP experiments suggested that PLD2 and ATP synthase interact, but did not indicate whether the interaction was direct or part of a larger protein complex. Conclusive experiments to determine direct protein interactions require purified proteins and since ATP synthase is a multiunit complex of over 500 kDa with multiple, genetically independent subunits, purification is impractical (Boyer, 1997). Therefore, we focused on investigating a physiological role for PLD in regulating intracellular ATP.

In order to measure intracellular ATP, we optimized the use of a luciferase-based bioluminescence assay. Luciferin is a substrate for the luciferase enzyme and in the presence of ATP, luciferase oxidizes luciferin to produce oxyluciferin, adenosine monophosphate (AMP), and light (Deluca and McElroy, 1978). Cells are lysed in a buffer compatible with luciferase activity and lysate is added to a reaction mixture containing luciferase and saturating concentrations of luciferin. Therefore, the amount of light emitted during the reaction, as measured by a luminometer, is proportional to the concentration of ATP in the sample and can be quantified using a standard curve of known ATP concentration.

Our initial experiments examined the effects of small-molecule PLD inhibitors on ATP levels in HEK293-TREx-EGFR cells. Under normal growth conditions, treatment of HEK293-TREx-EGFR cells with PLD inhibitors failed to reproducibly alter ATP levels at various time points tested. Since PLD activity remains low in most non-tumorigenic, non-stimulated cells, we decided to investigate whether conditions known to stimulate PLD activity also stimulate changes in intracellular ATP in a PLD-dependent manner. Multiple

cell lines were screened for an increase in intracellular ATP following EGF stimulation and included various GBM, renal, breast, and lung cancers along with HEK293 cells. For the screen, cells were seeded in complete growth media and allowed to attach for 24 hours. The following day, cells were washed and cultured in serum-free media to remove traces of EGF present in serum. Cells were treated with 100 ng/ml EGF for time points ranging from 30 minutes to 4 hours to determine an optimal time point for measuring changes in intracellular ATP. EGF stimulated an increase in intracellular ATP in multiple cell lines with one of the most robust increases measured in the U87MG cell line (Figure 8). In order to determine if PLD activity was required for the EGF stimulation of PLD activity, U87MG cells were pretreated with the VU0359595 PLD inhibitor for 30 minutes and then stimulated with 100 ng/ml EGF for 4 hours. PLD inhibition prevented the increase in intracellular ATP following EGF stimulation (Figure 9), suggesting that PLD activity is required for EGF-stimulated increases in ATP. Unfortunately, the U87MG cells were highly variable in this response, and EGF failed to increase intracellular ATP levels in several experiments. When EGF did elicit an ATP response, PLD inhibition consistently prevented the EGF-stimulated increase in intracellular ATP. Due to the highly variable nature of the EGF-response, we assayed other growth factors including PDGF, insulin, and insulin-like growth factor 1 using the same paradigm as EGF. None of the other individual growth factors elicited an ATP response in the U87MG cells (data not shown).

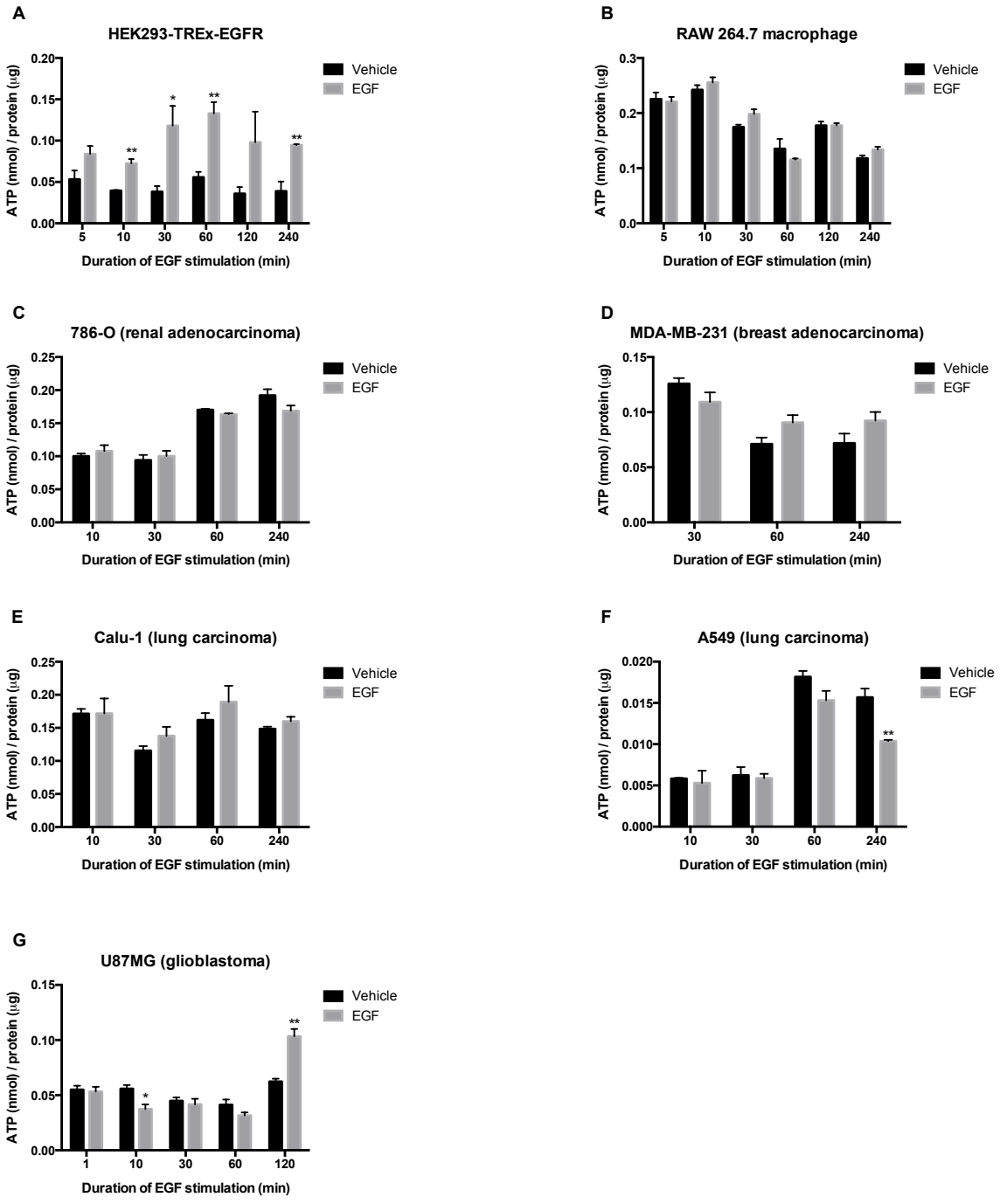

Figure 8. Screen of various cell lines for EGF-stimulated changes in intracellular ATP. Indicated cell lines were seeded to achieve approximately 60% confluency 24 hours after plating. Cells were deprived of serum overnight and stimulated with 100 ng/ml EGF for the indicated lengths of time before cell lysis and ATP quantification. * $p < 0.05$, ** $p < 0.01$, unpaired Student's t-test comparing vehicle to EGF stimulation for each time point.

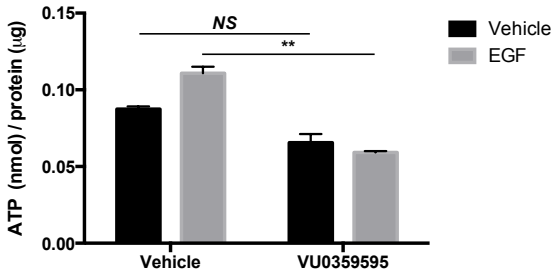

Figure 9. PLD mediates the EGF-stimulated increase in intracellular ATP. U87MG cells were serum-starved overnight and treated with 10 μM VU0359595 for 30 min before a 4-hour stimulation with 100 ng/ml EGF. ATP levels were quantified following EGF stimulation and normalized to total cellular protein in each sample. Shown is a representative experiment with triplicate samples. ** $p < 0.01$, *NS* – not significant, unpaired Student's t-test.

Serum stimulates increased intracellular ATP

Since we were unable to elicit a reproducible change in ATP levels with individual growth factors, we considered other stimulators of PLD activity. Serum contains many components including growth factors, cytokines, and amino acids that may function individually or synergistically to stimulate PLD activity and ATP synthesis. As such, previous studies have shown that serum stimulates PLD activity (Fang et al., 2001) and ATP synthesis (Hahn-Windgassen et al., 2005) in model systems. Various cell lines were screened for ATP changes following serum stimulation. Representative glioblastoma, breast, lung, and renal cancer lines along with HEK293-TREx cells were cultured in serum-free media overnight and stimulated with 20% fetal bovine serum (FBS) for durations ranging from 30 minutes to 4 hours. Multiple cell lines responded to serum stimulation by increasing intracellular ATP and the 4-hour serum stimulation time point resulted in the most robust ATP stimulation among the cell lines assayed (Figure 10).

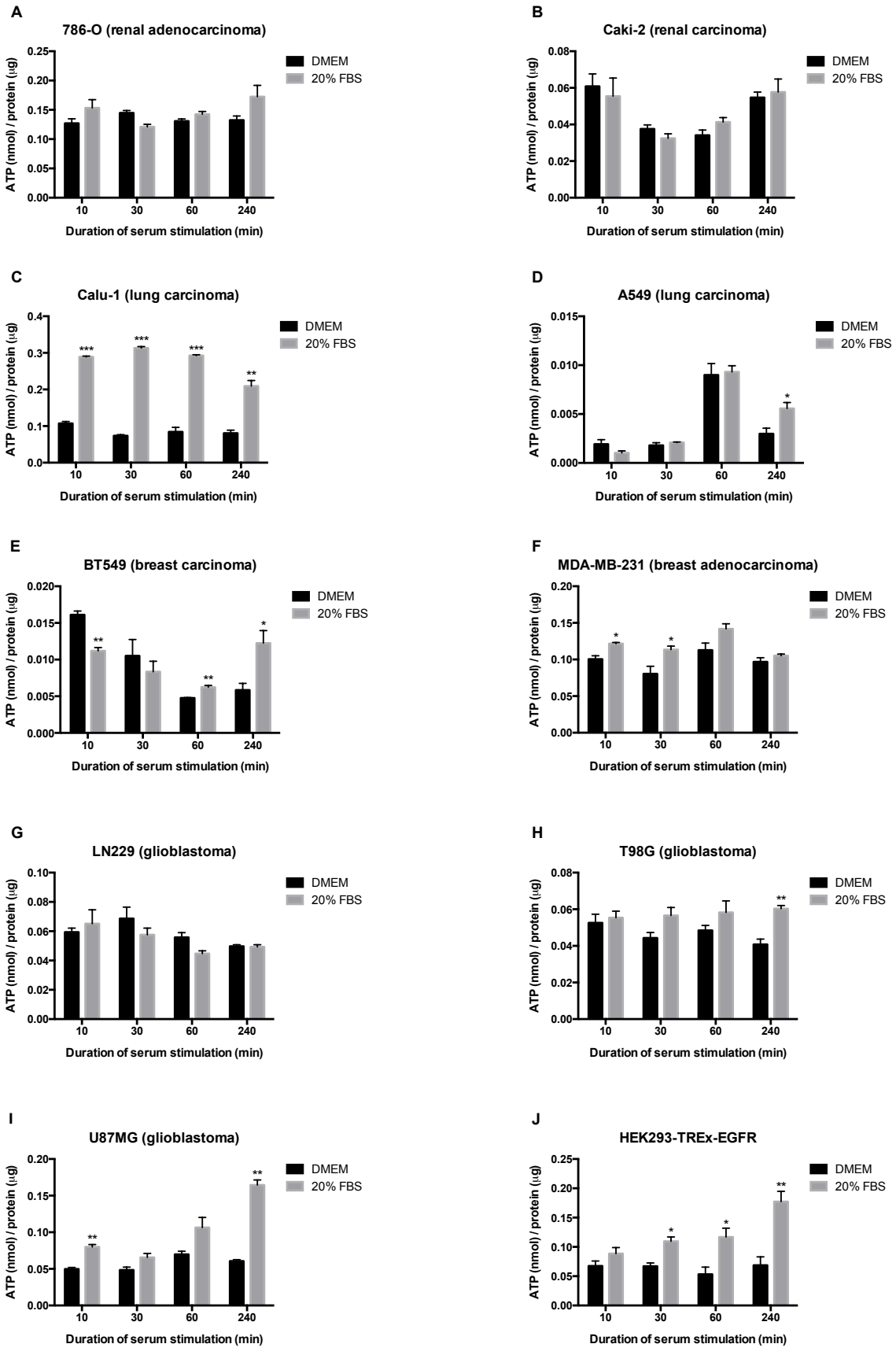
A
786-O (renal adenocarcinoma)
B
Caki-2 (renal carcinoma)
C
Calu-1 (lung carcinoma)
D
A549 (lung carcinoma)
E
BT549 (breast carcinoma)
F
MDA-MB-231 (breast adenocarcinoma)
G
LN229 (glioblastoma)
H
T98G (glioblastoma)
I
U87MG (glioblastoma)
J
HEK293-TREx-EGFR
DMEM
20% FBS
ATP (nmol) / protein (µg)
Duration of serum stimulation (min)
10
30
60
240

Figure 10. Screen of various cell lines for serum-stimulated changes in intracellular ATP. Indicated cell lines were seeded to achieve approximately 60% confluency 24 hours after plating. Cells were deprived of serum overnight and stimulated with 20% FBS for the indicated lengths of time before cell lysis and ATP quantification. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ unpaired Student's t-test comparing vehicle to serum stimulation for each time point.

PLD mediates the serum-ATP response in glioblastoma and breast cancer lines

In order to determine if the serum-induced ATP increase required PLD activity, we pretreated cells with PLD inhibitors for 30 minutes before stimulating with FBS and measuring ATP. Three cell lines demonstrated a dependence on PLD for the serum-induced ATP increase: U87MG and U118MG glioblastoma cells and BT549 breast cancer cells (Figure 11A-C). In these cell lines, PLD inhibitors suppressed the serum-stimulated ATP increase but did not change basal ATP levels. The parental HEK293-TREx line also responded to serum stimulation, but demonstrated a different pattern of ATP changes following PLD inhibition. Unlike the GBM and breast cancer lines, basal ATP levels consistently decreased following PLD inhibitor treatment in HEK293-TREx cells whereas the fold increase in the serum-stimulated ATP response remained unchanged (Figure 11D). The differences in ATP responses between the various cell lines suggested that PLD activity is required to meet the metabolic demands required for serum-induced proliferation in certain cancer cells.

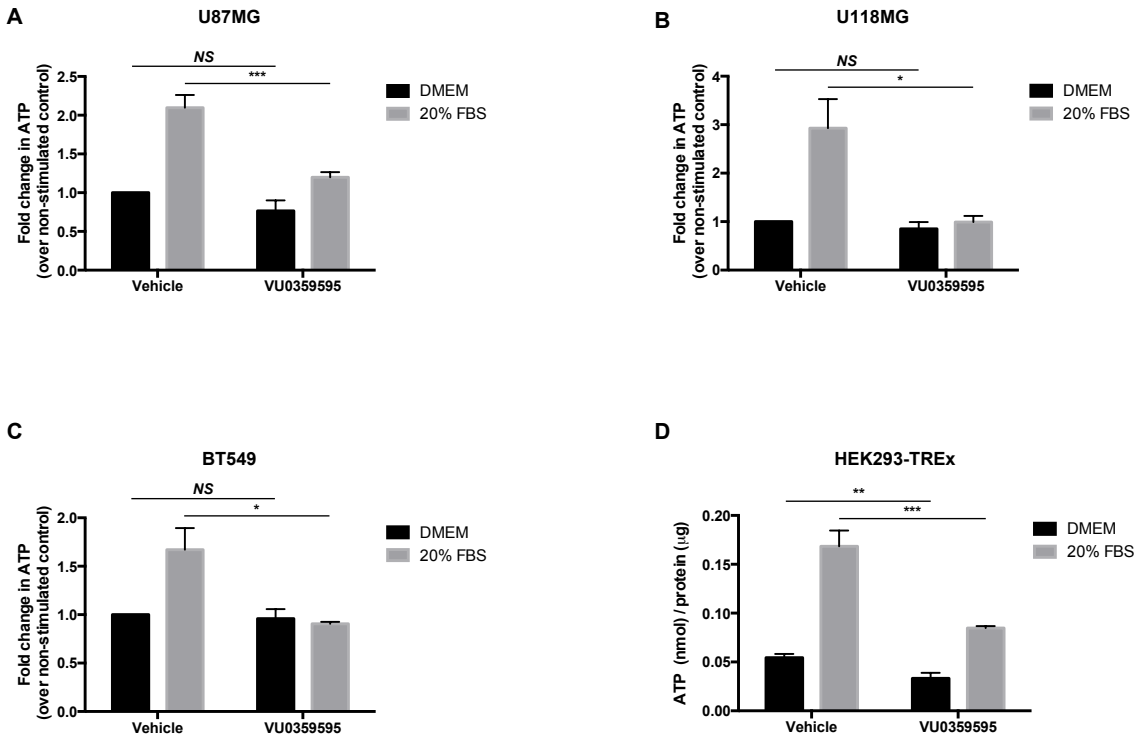

Figure 11. Serum stimulation of intracellular ATP requires PLD activity in multiple cancer lines. **A-C**. Cells were deprived of serum overnight, pretreated with 10 μM VU0359595 for 30 minutes, then stimulated with 20% FBS for 4 hours before ATP extraction and quantification. Data is presented as the fold change in ATP over non-stimulated controls for n=6 independent experiments for each cell line. * $p < 0.05$, *** $p < 0.001$, *NS* – not significant, paired Student's t-test. **D.** HEK293-TREx cells were treated as in (A-C). Shown is a representative experiment with triplicate samples. ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test.

To further support a role for PLD in regulation of cancer cell bioenergetics, we extended our analysis to a more relevant model system. Although the U87MG and U118MG cells are well-established GBM lines, we wanted to extend our study of the regulation of cancer cell bioenergetics by PLD in a more disease relevant system. As will be further discussed in chapter III, stem cells can be isolated from patient biopsies by sorting for the CD133 surface antigen and these stem cells phenocopy the patient's original tumor when injected into nude mice (Singh et al., 2004). Unlike the other cell lines discussed previously, these cells require growth in media containing a serum

substitute (B27) and a cocktail of EGF and FGF. In order to mimic the experimental paradigm established with the other GBM lines, we deprived the stem cells of B27 and growth factors overnight before pretreating with PLD inhibitors and stimulating with complete media containing B27, EGF, and FGF for 4 hours. The growth factor cocktail stimulated an increase in intracellular ATP in the GBM stem cells as in the established cell lines in a PLD-dependent manner (Figure 12). The dependence on PLD in a cancer model system that more closely resembles a primary tumor further supports a cancer-dependent role for PLD in regulating bioenergetics.

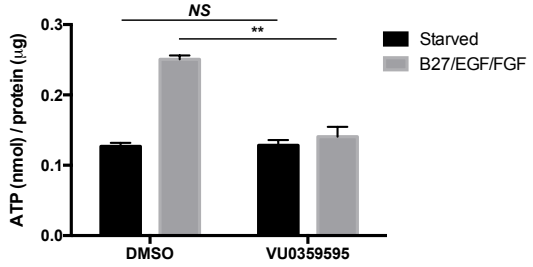

Figure 12. PLD activity is required for the serum-induced increase in intracellular ATP in CD133+ glioma stem cells. CD133+ glioma stem cell clone 4302 was seeded onto tissue culture plates coated with 0.5% laminin in complete growth media. Cells were cultured in neurobasal medium lacking growth supplements for approximately 16 hours prior to a 30 minute pretreatment with 10 μM VU0359595. Cells were subsequently stimulated with complete growth media containing B27, EGF, and FGF for 4 hours before ATP measurement.

Excluding off-target effects of VU0359595 on the serum-ATP response

In order to exclude off target effects of VU0359595, we first titrated the concentration of inhibitor needed to fully inhibit the serum-ATP response. VU0359595 is a PLD1-preferring compound with a cellular IC_{50} value of 3.7 nM for PLD1 and 6.4 μM for PLD2 (Lewis et al., 2009). In the previous assays, VU0359595 was used at a relatively high concentration of 10 μM, a concentration that would completely inhibit PLD1 and partially inhibit PLD2. To determine the concentration of VU0359595 needed

to completely inhibit the serum-ATP response in U118MG cells, cells were deprived of serum overnight and then pretreated with various concentrations of PLD inhibitors before a serum stimulation the following day. VU0359595 concentrations as low as 100 nM completely inhibited the serum-ATP response in U118MG cells (Figure 2-13). Although the concentration response curves remain to be generated for the serum stimulation of PLD activity in the U87MG cells, the concentration response curve for PLD inhibition of the serum-ATP stimulation in the U118MG remarkably resemble the curve for PLD1 inhibition in our Calu-1 model system (Lewis et al., 2009). Although not conclusive, the low concentration of VU0359595 needed to inhibit the serum-ATP response argues strongly for specific inhibition of PLD as the mechanism for VU0395595 inhibition of the serum-ATP response.

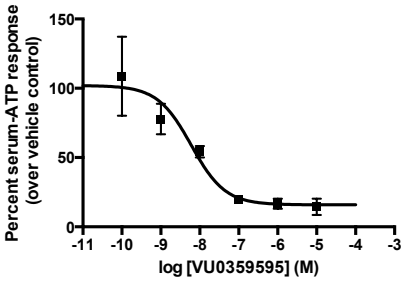

Figure 13. VU0359595 concentration response curve for the serum-ATP response in U118MG cells. Cells were deprived of serum overnight and then pretreated with the indicated concentration of VU0359595 for 30 minutes before a 4-hour stimulation with 20% FBS. Data is presented as the percent inhibition of the serum stimulated ATP response due to PLD inhibition relative to vehicle control.

After establishing the specificity of our PLD inhibitor in the serum-ATP response, we silenced expression of either PLD1 or PLD2 to determine if ablation of PLD protein mimicked the small molecule inhibitor effect. U118MG or U87MG glioblastoma cells were transfected with PLD1, PLD2, or a combination of PLD1 and PLD2 siRNA at a final concentration of 100 nM. Forty-eight hours after transfection, cells were washed and

cultured in serum-free media overnight and cells were stimulated with 20% FBS for 4 hours approximately 72 hours after the siRNA transfection. Silencing of either PLD1 or PLD2 significantly decreased the fold increase in intracellular ATP following serum stimulation (Figure 2-14). Together, these data suggest that both PLD1 and PLD2 are required to mediate the signaling events necessary to increase ATP levels following extracellular stimuli and that the effects of VU0359595 on ATP production are due to inhibition of PLD.

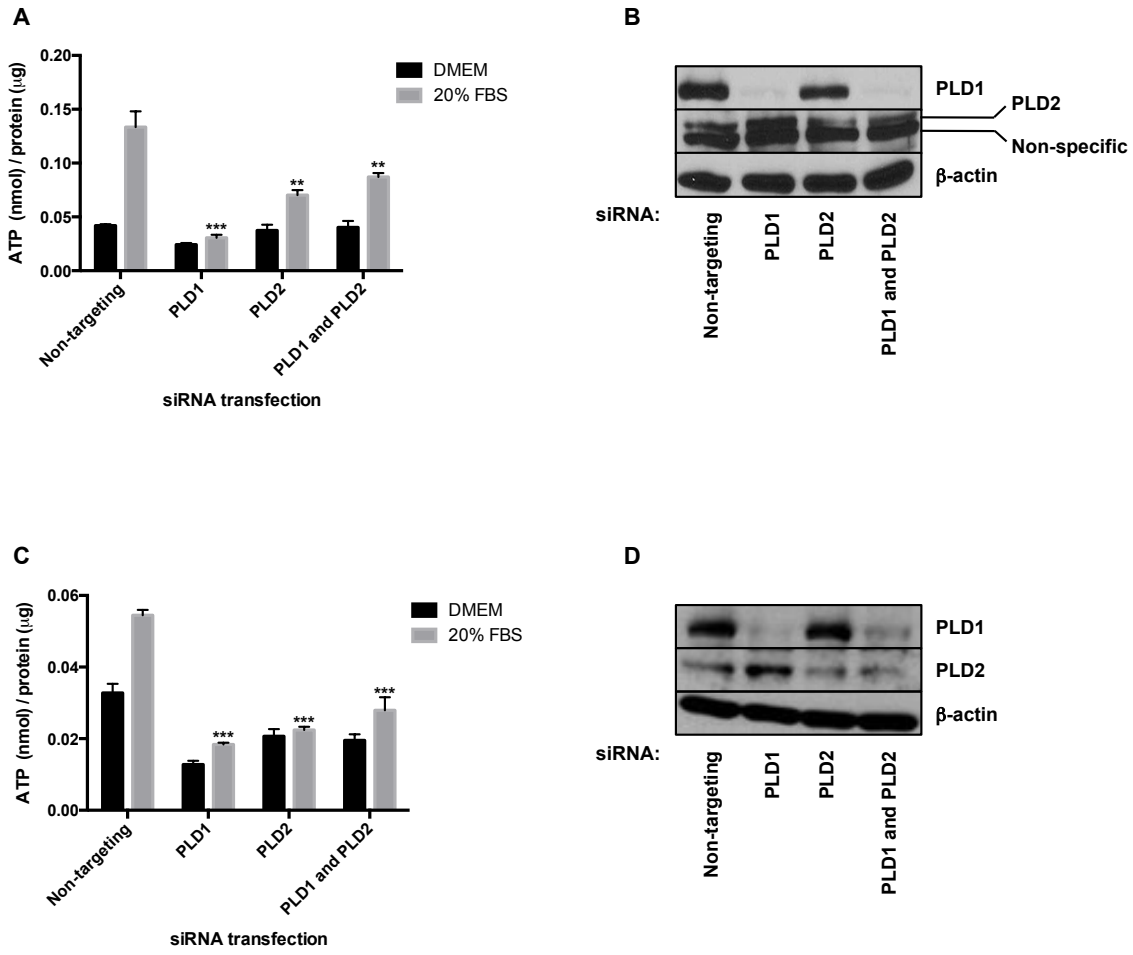

Figure 14. siRNA silencing of PLD1 and PLD2 decreases the serum-induced increase in intracellular ATP in GBM cells. **A**. U87MG cells were transfected with 100nM siRNA targeting either PLD1 or PLD2 as indicated. 48 hours after transfection, cells were deprived of serum for an additional 24 hours. Cells were then stimulated with 20% FBS for 4 hours before lysis and ATP measurements. **B**. Representative immunoblot of siRNA knockdown of PLD1 or PLD2. Note, the commercial PLD2 antibody recognizes a non-specific band immediately below PLD2 in the U87MG cell line. **C and D**. U118MG cells were treated as in (A) and (B). ** $p < 0.01$, *** $p < 0.001$, one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test comparing FBS-stimulated ATP levels in the siRNA treated samples to the non-targeting controls.

Characterization of the molecular mechanisms for bioenergetic regulation by PLD

The increase in intracellular ATP following serum stimulation could result due to an increase in ATP synthesis or a decrease in metabolic demand. Given the mitogenic and proliferative properties of serum, the increase in ATP is likely due to increased

biosynthesis of ATP to fuel processes required for cell growth. Multiple pathways exist to generate requisite ATP including glycolysis and mitochondrial oxidative phosphorylation. In order gain insight into the pathways modulated by PLD, we used chemical tools to dissect the two ATP-generating pathways. 2-deoxyglucose (2-DG) is a glucose analog that lacks a hydroxyl group in the 2 position of the glucose ring. Therefore, 2-DG passes through the glucose transporter and is phosphorylated by hexokinase but cannot continue into the glycolysis pathway since the next enzymatic step is isosomerization of glucose to fructose 6-phosphate by phosphoglucose isomerase. Thus, the charged 2-deoxyglucose 6-phosphate is trapped within the cell and inhibits further glycolysis by product inhibition of hexokinase, the rate-limiting step of glycolysis (Wick et al., 1957). Oligomycin is a bacterial macrolide that blocks the function of ATP synthase in the mitochondria and inhibits mitochondrial ATP production (Guerrieri et al., 1976). By binding to and occupying the pore of the F_O component of ATP synthase, oligomycin inhibits electron-transport-chain derived H^+ ions from passing down their electrochemical gradient through the ATP synthase complex and the energy source for ATP catalysis from ADP and inorganic phosphate is thus uncoupled.

To determine which pathways generated serum-stimulated ATP, we treated serum-starved U118MG or HEK293 cells with 2-DG or oligomycin for 2 hours before a 30-minute VU0359595 pretreatment. After pretreatments, cells were stimulated with 20% FBS for 4 hours as in previous experiments. Cells were collected for ATP analysis, and media was saved for lactate analysis as a measurement for glycolytic activity. Lactate is produced when the end product of glycolysis, pyruvate, is reduced by the lactate dehydrogenase complex (LDH). Lactate production is frequently used as a measure of glycolytic activity. A common method for measuring lactate concentrations in cell media uses a two-step enzymatic reaction. In the first reaction, lactate is oxidized to pyruvate by LDH in the presence of NAD^+. In the second reaction, a diaphorase enzyme reduces

a tetrazolium salt such as INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) using the newly produced NADH to generate a formazan dye which absorbs light at 490 nM (Wolterbeek and van der Meer, 2005). Thus, the absorbance of the formazan dye is directly proportional to the amount of lactate present in the sample. Measurement of mitochondrial oxygen consumption is more difficult and investigators have historically used a Clark electrode to measure oxygen levels in a sample containing actively respiring mitochondria. As samples must be constantly stirred to maintain equilibrium, this technique becomes impossible for most monolayer cell cultures. New techniques, such as the use of oxygen-sensitive fluorescent probes, show promise for measuring mitochondrial respiration in cultured cells. Cells are grown on microtiter plates and loaded with a fluorescent dye that is quenched by O_2. As cells respire and O_2 levels decrease, fluorescence increases (Hynes et al., 2005). In our hands, these dyes were not sensitive enough to detect respiration in U118MG or HEK293 cells so measurement of metabolic activity attributable to individual pathways was limited to lactate production and glycolysis.

The ATP measurements following 2-DG or oligomycin treatment were highly variable in all of the cell lines tested. In some experiments, 2-DG dramatically decreased overall ATP levels whereas oligomycin had little to no effect. In other experiments, individual inhibitor treatment did not alter basal ATP levels. Additionally, serum stimulated an increase in ATP even in the presence of individual metabolic inhibitors. Together these results indicate that these cells are capable of using both ATP-generating pathways and that serum-stimulation engages both glycolysis and oxidative phosphorylation.

Although the ATP results were inconclusive for determining the pathway regulated by PLD, the lactate measurements were much more reproducible and provided several insights into the ATP biosynthetic pathways engaged by serum

stimulation (Figure 15). First, 2-DG treatment abolished lactate generation in both cell lines as would be expected from glycolysis inhibition and oligomycin treatment increased lactate production, presumably by inducing an upregulation of glycoloytic activity. Second, lactate levels did not increase following serum stimulation in either cell line, suggesting that glycolysis was not the predominant pathway of ATP generation following serum stimulation. We observed divergent responses to basal lactate levels between HEK293-TREx and U118MG cells where VU0359595 selectively decreased basal lactate only in the HEK293-TREx cells (Figure 15A). The decrease in basal lactate levels in HEK293-TREx cells following VU0359595 treatment correlates with the decrease in basal ATP we measured following PLD inhibitor treatment (Figure 11D) and suggests that HEK293-TREx cells utilize glycolysis for basal ATP production more than U118MG cells. Finally, VU0359595 completely inhibited the elevation in oligomycin-induced lactate production in both cell lines, which suggests that PLD may somehow function to promote the switch from oxidative phosphorylation to glycolysis. In order words, PLD may serve to act as a sensor of cellular energy homeostasis at the level of the mitochondria.

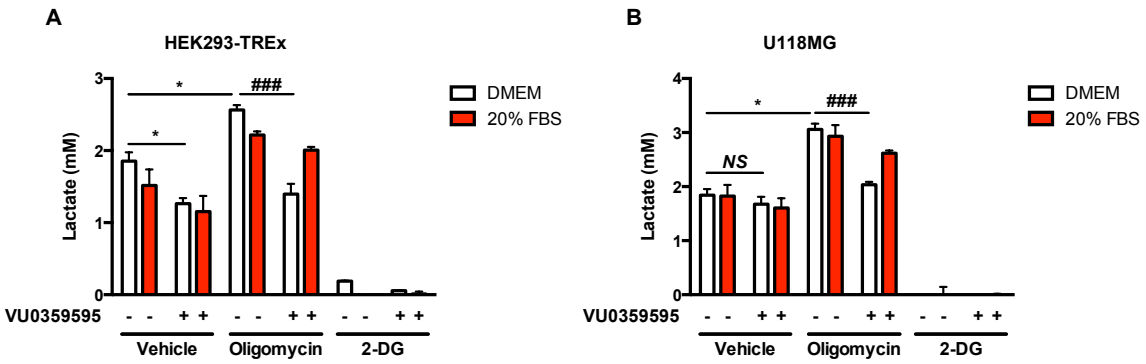

Figure 15. Serum stimulation of lactate production in the presence of metabolic and PLD inhibitors. **A**. HEK293-TREx cells were deprived of serum overnight before treatment with either 1 μM oligomycin or 10 mM 2-DG for 2 hours. Cells were treated with 10 μM VU0359595 for the final 30 minutes of the 2-hour pretreatment. Cells were finally stimulated with 20% FBS for 4 hours before media was collected for lactate measurement. **B**. U118MG cells were treated similarly to HEK293-TREx cells. Shown are representative experiments with triplicate samples. * $p < 0.05$, *** $p < 0.001$, one-way ANOVA with Tukey's multiple comparison test comparing indicated sample conditions. ### $p < 0.001$, unpaired Student's t-test comparing vehicle to VU0359595 treatment within the oligomycin condition. *NS* – not significant.

Investigation of PLD as a sensor of bioenergetic homeostasis

Treatment of cells with oligomycin would likely decrease intracellular ATP and upregulate compensatory pathways for ATP generation and the experiment in Figure 15 suggested that PLD was a required component of those compensatory signaling pathways. We therefore hypothesized that PLD was acting as a sensor of cellular bioenergetic status and transduced signals in response to intracellular energy fluctuations. In order to test this hypothesis, we performed exogenous PLD activity assays using recombinant, purified PLD2 in the presence of various concentrations of ATP, ADP, and AMP to determine if PLD activity was directly regulated by adenine nucleotides. High concentrations of ATP and ADP inhibited PLD2 activity whereas AMP had no effect on PLD2 activity at the same concentrations (Figure 16). Although the concentrations of ATP and ADP required to inhibit PLD activity were in the millimolar range, the concentrations of intracellular ATP are similarly high (Beis and Newsholme,

1975). These results suggest that high basal levels of intracellular ATP might inhibit PLD activity. As ATP levels decrease, either through metabolic poison or through increased metabolic demand such as proliferation following serum stimulation, PLD activity would increase and initiate signaling cascades to generate ATP.

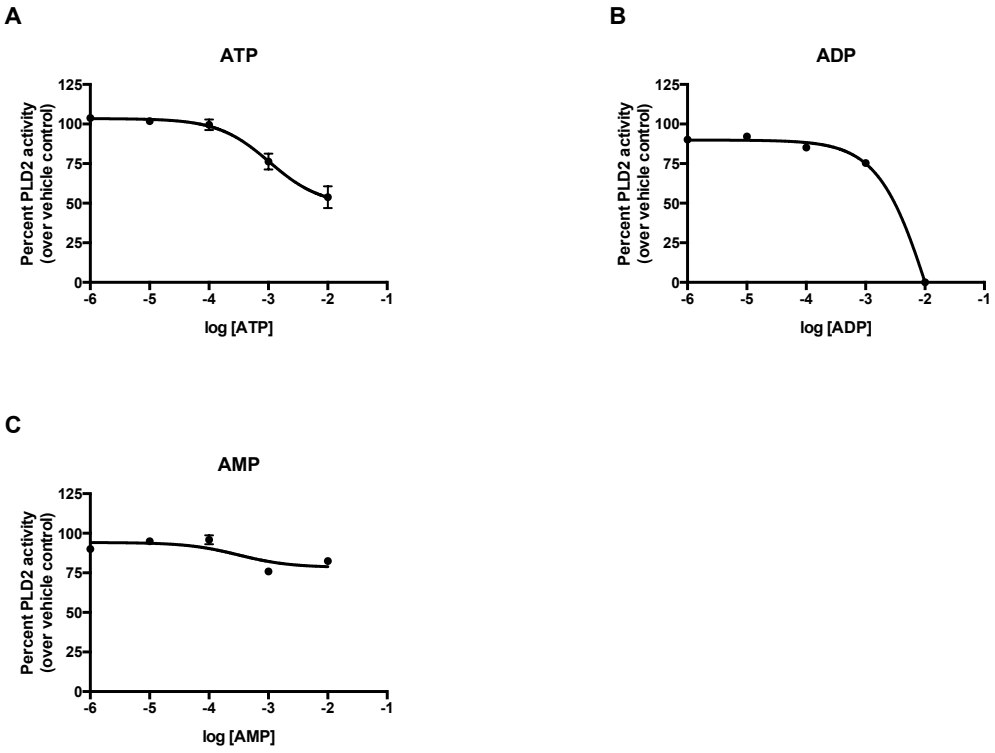

Figure 16. Concentration response curves for adenine nucleotides on PLD2 activity. PtS-PLD2 was purified from *Sf21* insect cells and activity was measured as choline release from [^{3}H]PC in the presence of the indicated concentrations of ATP, ADP, or AMP. Data is presented as the percent PLD2 activity remaining in the adenine nucleotide samples relative to vehicle control.

Characterization of serum factors responsible for stimulating ATP in GBM cells

Serum is a complicated mixture of growth factors, cytokines, fatty acids, and other nutrients such as amino acids and carbohydrates including the individual growth factors we assayed for ATP stimulating activity. Determining the component(s) of serum responsible for stimulating PLD activity and increasing intracellular ATP has potential

therapeutic value if a specific signaling pathway can be targeted in cancer cells. In order to determine if low molecular weight factors such as glucose or amino acids were stimulating the ATP response, we dialyzed FBS using a 3500 molecular weight cutoff membrane against phosphate buffered saline overnight. Successful dialysis was confirmed by measuring lactate concentrations in FBS before and after dialysis. Both dialyzed and non-dialyzed FBS stimulated an ATP increase in the U118MG cell line, but VU0359595 did not inhibit the serum-ATP response when cells were stimulated with dialyzed FBS (Figure 17A). Additionally, stimulation with non-dialyzed FBS did not increase lactate production in HEK293-TREx or U118MG cells, in agreement with previous experiments (Figure 15). By contrast, dialyzed FBS stimulated a robust increase in lactate production in the U118MG cell line (Figure 17B).

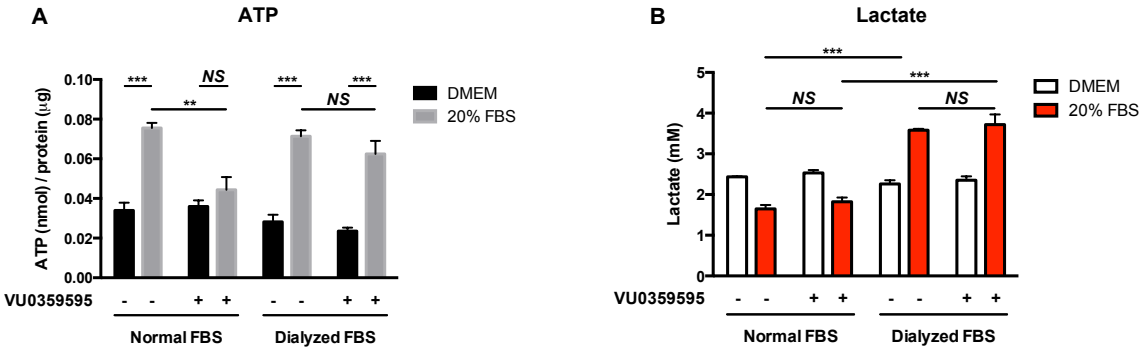

Figure 17. ATP and lactate production in response to dialyzed and non-dialyzed FBS in U118MG cells. Cells were deprived of serum for approximately 16 hours before a 30 minute pretreatment with 10 mM VU0359595. Cells were subsequently stimulated with 20% non-dialyzed FBS or FBS dialyzed overnight using a 3500 molecular weight cutoff dialysis membrane. After 4 hours, media was saved for lactate measurements and cells were lysed for ATP measurements. Shown is a representative experiment with triplicate samples. ** $p < 0.01$, *** $p < 0.001$, *NS* – not significant, one-way ANOVA with Tukey's multiple comparisons test comparing indicated conditions.

Several conclusions can be drawn from analyzing the effects of dialyzed FBS on the ATP and lactate levels in U118MG cells. Both normal and dialyzed serum treatments stimulated ATP to the same magnitude. However, the elevation in lactate following

dialyzed FBS treatment suggests that glycolysis is the primary pathway for ATP generation under this treatment condition. PLD inhibition does not block the ATP response to dialyzed FBS, supporting the notion that PLD regulates bioenergetics at the level of the mitochondria and not through regulating glycolysis. One potential explanation for the switch from oxidative phosphorylation to glycolysis following dialysis of FBS is the removal of lactate from FBS following the dialysis procedure. LDH is known to demonstrate product inhibition where high concentrations of lactate reduce LDH activity (Karlsson et al., 1974). With lactate removed from FBS, glycolytic activity would increase with LDH no longer inhibited due to high levels of extracellular lactate. Taken together, the data in this chapter suggest that PLD enzymes interact with metabolic enzymes and regulate ATP production, and may possibly govern the fluctuation between oxidative phosphorylation and glycolysis to provide requisite energy needed during times of cell proliferation. Elucidating the exact molecular mechanisms of the PLD-ATP pathways should be the subject of future investigations.

Materials and methods

Cell culture

U87MG, U118MG, and BT549 cells (ATCC) and HEK293-TREx (Life Technologies) were maintained in Dulbecco's modified eagle's medium (DMEM) (Life Technologies) + 10% FBS (Atlanta Biologicals) + 1% Penicillin/Streptomycin (P/S) (Life Technologies). Inducible, HEK293-TREx derived cell lines were maintained in DMEM + 10% tetracycline-free FBS (Atlanta Biologicals) + 1% P/S. CD133+ glioma stem cells were cultured as described (Wang et al., 2010a). Stem cells were maintained in neurobasal media containing glutamine, B27, sodium pyruvate (all from Life Technologies), 20 ng/ml fibroblast growth factor and epidermal growth factor

(Peprotech). All human cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Plasmid production

myc-PLD2 was created by PCR amplification of the PLD2 open reading frame (PLD2 cDNA was a generous gift from Dr. David Lambeth at Emory University) primer: 5'- ataagaatgcggccgccatggaacaaaaactcatctcagaagaggatctgATGACGGCGACCCCTGAG -3', reverse primer: 5'- gctctagaCAACTATGTCCACACTTCTAG -3'). myc-PLD2 was ligated into the NotI/XbaI restriction sites of pcDNA5/TO (Life Technologies). See chapter III for details on the construction of all other plasmids.

Transfection

Mammalian cells were transfected using Fugene 6 (Roche) according to the manufacturer's instructions using the standard volumes of transfection reagent and DNA quantities. Cells were harvested approximately 48 hours post transfection. Stable HEK293-TREx cell lines were generated by transfection of indicated construct as per the manufacturer's instructions. 48 hours after transfection, cells were split into media containing 100 μg/ml hygromycin B (Life Technologies). Cells were grown until colonies developed. Individual colonies were harvested by aspiration into a 200 μλ pipette tip under a microscope and expanded as monoclonal colonies. Cells were eventually screened for stable transfection by immunoblotting.

Immunoblotting

Lysates were prepared by incubating cell pellets in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 5 mM NaF, 1 mM DTT, and

Roche complete protease inhibitor cocktail for 30 minutes at 4 °C. Protein concentrations were measured using the Bio-Rad protein assay reagent. Myc antibody (clone 9E10) was from AdD Serotec). PLD1, ATP synthase, HSP90, and ANT2 antibodies were from Santa Cruz. Strep-tag antibodies were from Qiagen. PLD2 antibody was from Abgent.

Immunoprecipitation

Cells were resuspended in lysis buffer (see Immunoblotting section) and lysed with three freeze/thaw cycles in dry ice/ethanol, drawing lysate through a 25G syringe needle between cycles. Clarified (10,000 × *g* for 10 min) lysates were pre-cleared with protein-G agarose (Millipore) and immunoprecipitating antibodies were incubated with lysate overnight. Complexes were captured using protein-G agarose, washed three times in lysis buffer, then eluted by boiling in 2× SDS-PAGE loading buffer.

Proteomic analysis

Proteins were delivered as TCA precipitates to the Vanderbilt proteomics core and peptides were resolved and fragmented according to previously established methods (Link et al., 1999; McDonald et al., 2002). Peptide sequences were assigned to tandem mass spectra using the IDpicker software (Ma et al., 2009).

ATP measurement

Cells were seeded to achieve approximately 60% confluence the next day. After allowing cells to adhere for 24 hours, cells were washed twice in phosphate buffered saline (PBS) and then cultured for approximately 16 hours in serum free DMEM. Where indicated, metabolic inhibitors or PLD inhibitors were added 2 hours or 30 minutes, respectively, before stimulation with growth factors (all from Life Technologies) or 20% FBS. Cells were washed once then scraped in ice-cold PBS. Cells were collected by

centrifugation at 1,000 × *g* at 4 °C for 5 minutes. Pellets were resuspended in 60 μl extraction buffer (50 mM Tris pH 7.75, 4 mM EDTA) and boiled for 5 minutes. After boiling, extracts were cooled on ice then centrifuged at 18,000 × *g* for 5 minutes. 40 μl of extract was transferred to a new tube and protein concentrations were measured from undiluted extracts using the Bio-Rad protein assay. Extracts were then diluted 1:10 in extraction buffer and 50 μl of the dilution loaded into 96-well plates. Luciferase activity was measured by automated injection of 50 μl luciferase reagent (Roche, ATP Bioluminescence Assay Kit CLS II) per well and then luminescence was measured for 10 seconds in 0.1 second intervals following a 2 second delay after the addition of luciferase reagent. Luminescence was integrated over the 10-second collection time and used to calculate [ATP] based on the luminescence of a standard ATP curve. ATP was calculated as nanomoles ATP per microgram protein in each sample.

Lactate measurement

Following experimental manipulation of cells for ATP measurement, media was saved and frozen at -80°C until time of assay. Samples were diluted in DMEM to obtain values within the linear range of the assay. Lactate measurements were performed using the L-Lactate Assay Kit I from Eton Biosciences and [lactate] determined from a standard curve. Blank samples containing 20% FBS or DMEM alone were used for background subtraction from stimulated and non-stimulated samples, respectively.

Exogenous PLD activity assay

In vitro PtS-PLD2 activity was measured as choline release from [^{3}H]PC-containing phospholipid vesicles as described previously (Brown et al., 1993). Stock concentrations of adenine nucleotides were made from powder in PLD activity assay

buffer and diluted to the final concentration indicated in the figures. ATP was obtained from Avanti Polar Lipids. ADP and AMP were obtained from Sigma.

Purification of PLD2

Detailed methods for purification of PLD2 are found in chapter III.

CHAPTER III

PHOSPHOLIPASE D2 MEDIATES SURVIVAL SIGNALING THROUGH DIRECT REGULATION OF AKT IN GLIOBLASTOMA CELLS

Introduction

Glioblastoma multiforme, the most common and aggressive glioma, is a highly lethal type of brain tumor with poor patient prognosis. Despite advances in imaging and neurosurgery over the past 30 years, GBM remains one of the most difficult tumors to manage with a median survival time of approximately 14 months following diagnosis (Stupp et al., 2005). Treatment options are limited and invasive, typically including a combination of surgical resection, radiotherapy, and adjuvant chemotherapy (DeAngelis, 2001). Less invasive, small molecule therapies for GBM have been met with limited success due, in part, to the poor blood-brain-barrier penetrance of current chemotherapeutics that limits their access to GBM tumors. Moreover, the aggressive nature of GBM, its genetic variability, and antineoplastic drug resistance further curbs the effectiveness of small molecule inhibitors to treat the disease. The combination of these clinical obstacles contributes to the comparatively short survival times and patient death rate (Eramo et al., 2006). Thus, the field requires a more thorough understanding of GBM signaling and metabolic pathways to develop novel treatment options.

Among the most frequently deregulated pathways in GBM are components of the PI3K/Akt pathway (Cheng et al., 2009). Activated PI3K, either by cell-surface receptor stimulation or mutation to a constitutively active form, results in PIP_3 production and subsequent initiation of signaling cascades by recruiting a variety of molecules containing lipid-binding domains to membranes (Cantley, 2002). The serine/threonine kinase Akt was identified as the eukaryotic homolog of the retroviral oncogene v-Akt,

which becomes activated following PI3K generation of PIP_3 (Bellacosa et al., 1991; Franke et al., 1995). Akt mediates a variety of intracellular functions critical to oncogenic processes, including cell growth, proliferation, metabolism, and survival (Manning and Cantley, 2007). Mutations that result in PI3K activation, such as constitutive growth factor receptor activation (Libermann et al., 1985) or inactivation of PTEN (Haas-Kogan et al., 1998), the lipid phosphatase that hydrolyzes PIP_3, are common in GBM. While small-molecule inhibitors of the PI3K/Akt pathway hold promise in clinical trials for GBM (Furnari et al., 2007), global inhibition of the Akt isoenzymes results in side effects that limit their clinical potential (Yap et al., 2011).

In addition to PIP_3, other lipids such as PtdOH are known to mediate intracellular signaling events that are required for oncogenic processes. Multiple cancer types including breast, gastric, and renal cancers show elevated PLD activity compared to normal tissue (see Chapter I). Cells overexpressing PLD demonstrate increased AIG (Min, 2001), invasiveness (Park et al., 2009), and tumorigenesis in nude mice (Buchanan et al., 2005). Mechanistically, PLD and PtdOH regulate cytoskeletal rearrangement (Henkels et al., 2013a), angiogenesis (Chen et al., 2012), and expression of matrix metalloproteases (Park et al., 2009), which are all requirements for invasion and metastasis. The development of small molecule PLD inhibitors that decrease cancer cell invasiveness (Scott et al., 2009), along with the development of PLD knockout mice that show no overt negative phenotypes (Elvers et al., 2010; Oliveira et al., 2010), makes PLD a promising therapeutic target.

Recent reports have suggested a possible relationship between PLD and Akt involving both direct (Di Fulvio et al., 2008; Patel et al., 2010) and indirect (Toschi et al., 2009) mechanisms. Interestingly, PLD from *Neisseria gonorrhoeae* regulates human Akt kinase activity upon infection of cervical epithelial cells (Edwards and Apicella, 2006). In this chapter, we investigate the regulation of Akt by human PLD and demonstrate a

novel mechanism by which PtdOH activates Akt and mediates survival signaling in GBM cells. By targeting PLD, we explore novel treatment options for regulating Akt kinase activity for the treatment of human brain cancers.

Results

PLD2 activity is required for glioma viability following serum-withdrawal

Multiple cancer types require PLD and its product, PtdOH, for sustained survival under stress conditions (Foster and Xu, 2003). Serum-withdrawal, a known stimulus of PLD activity in multiple cancer cell lines (Zheng et al., 2006), is frequently used to simulate the harsh growth environments encountered by neoplastic cells prior to vascularization and restoration of nutrient supply within the tumor mass. Viability is compromised when normal cells are cultured in serum-depleted conditions. Cells with elevated PI3K/Akt activity, however, continue to proliferate under these harsh culture conditions (Sun et al., 1999). To investigate the role of PLD in GBM survival, we measured PLD activity following serum-withdrawal in the PTEN-null U87MG GBM cell line. Cells were grown overnight in complete growth media (DMEM with FBS) before growth in media lacking FBS for times ranging from 1 to 24 hours. Serum-withdrawal resulted in a time-dependent increase in PLD activity with the most robust activation observed after 16 hours and longer durations of serum-withdrawal did not further increase PLD activity (Figure 1A). By contrast, we did not observe increased PLD activity in the non-tumorigenic HEK293 line under the same conditions (Figure 1A), suggesting a cancer line specific PLD response. Serum deprivation leading to PLD activation in U87MG cells is consistent with published reports on other cancer cell lines showing similar trends (Zheng et al., 2006).

Two isoforms of PLD have been identified, PLD1 and PLD2 and each demonstrates distinct regulatory properties (see Chapter I). To better understand the role of each isoform in this stress pathway we explored which PLD isoform was preferentially activated following serum-withdrawal using both pharmacological and genetic tools. In the first approach, U87MG cells were serum deprived overnight and then treated with various concentrations of previously reported, isoform-preferring PLD inhibitors. VU0359595 is a 1,700-fold PLD1-preferring compound (Lewis et al., 2009) and VU0364739 is a 75-fold PLD2-preferring compound (Lavieri et al., 2010), as determined with cell-based assays designed to measure activity of individual PLD isoforms. In the U87MG cells, which express both PLD1 and PLD2, VU0359595 and VU0364739 attenuated PLD activity following serum-withdrawal with IC_{50} values of approximately 500 nM and 100 nM, respectively (Figure 1B). The five-fold greater potency of the PLD2-preferring compound suggests that the PLD2 isoform is responsible for the vast majority of PLD activity in these cells following serum-withdrawal, although the PLD1 isoform may partially compensate following acute inhibitor treatment. To further explore the contribution of individual isoforms to the total PLD activity, we utilized isoform-specific siRNA to knock down either PLD1 or PLD2 and measure PLD activity following overnight serum-withdrawal. Silencing of PLD2, but not PLD1, resulted in a significant decrease in PLD activity (Figure 1C), further implicating PLD2 as the predominant isoform in the serum-withdrawal response.

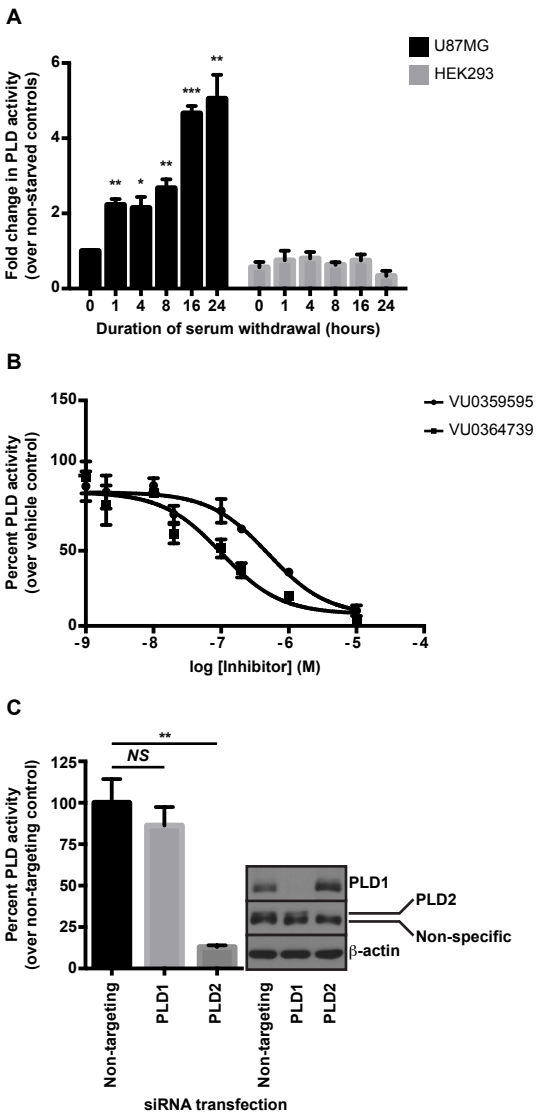

Figure 1. Serum-withdrawal stimulates PLD2 activity in GBM cells. **A**. Cells were seeded approximately 24 hours prior to washing and incubation in serum-free media for the indicated length of time. *n*-Butanol (*n*-butanol-d_{10}) was added 30 minutes prior to glycerophospholipid extraction and subsequent PtdBuOH quantification. **B**. U87MG cells were seeded as in (A) and serum was withdrawn for 24 hours. Cells were pretreated with inhibitors 30 minutes prior to measurement of PLD activity. Data is presented as the percent activity remaining after PLD inhibitor treatment relative to control. **C**. U87MG cells were transfected with siRNA targeting either PLD1 or PLD2 for 48 hours prior to a 24-hour serum starvation before measuring PLD activity. Note the PLD2 antibody recognizes a non-specific band of similar molecular weight to PLD2 and the band of interest is directly above the non-specific band. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, *NS* - not significant, unpaired Student's t-test. Error bars - standard error of the mean (SEM)

In order to determine if PLD activity was required for viability in U87MG cells following serum-withdrawal, we measured cell viability following overnight treatment with various concentrations of PLD inhibitors. U87MG cell viability decreased in a concentration-dependent manner (Figure 2A), consistent with concentrations needed to completely ablate PLD activity (Figure 1B), suggesting that complete suppression of PLD activity compromises viability in these cells. By contrast, treatment of HEK293 cells with PLD inhibitors resulted in significantly less cell death when compared to U87MG cells (Figure 2B), further implicating PLD as necessary for cancer cell survival.

Although U87MG cells are a well-characterized GBM line, we wanted to extend our study on PLD to a more disease-relevant model, namely cells isolated from biopsies of primary human GBMs. Glioma stem cells can be isolated from patient tumors by sorting for surface expression of the CD133 antigen. These stem cells are tumorigenic and phenocopy the patient's original tumor when injected into immunocompromised mice (Singh et al., 2004). Two glioma stem cell clones (Wang et al., 2010a), derived from individual patients, both showed reduced viability following PLD inhibitor treatment under growth factor starvation (Figure 2C). Anchorage-independent growth, the most important measure of tumorigenicity (Shin et al., 1975), was then assessed in these stem cells. Following PLD inhibitor treatment, GBM stem cells formed significantly fewer colonies than vehicle control samples in soft agar, even in the presence of growth factor supplements (Figure 2D). Together these results demonstrate that PLD activity is required for proliferation and survival in glioma cells.

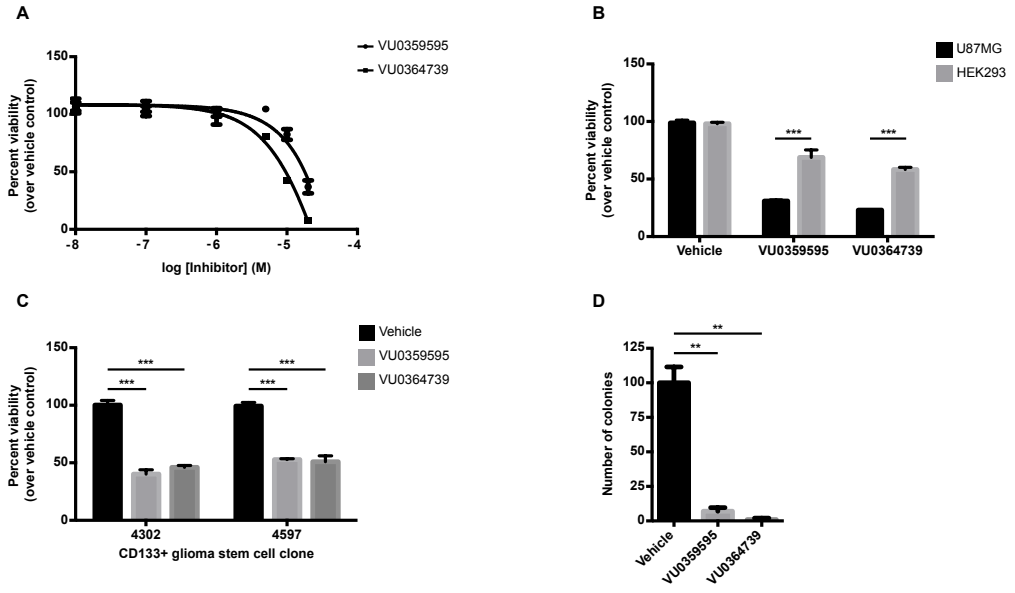

Figure 2. PLD activity is required for cell viability and AIG in GBM cells. **A**. Cells were treated with indicated concentration of PLD inhibitor for 24 hours in serum-free media. Following inhibitor treatment, viability was measured using the WST-1 reagent. **B**. U87MG or HEK293 cells were grown in complete growth media for 24 hours. Cells were then treated for 24 hours with 10 μM VU0364739 or 20 μM VU0359595 in serum-free DMEM. Viability was measured using the WST-1 reagent. *** $p < 0.001$, two-way ANOVA with Sidek's post-hoc test to compare viability between each cell line within each inhibitor treatment group. **C**. CD133+ glioma stem cells were seeded into 96-well plates in media containing growth supplements and laminin to facilitate adhesion. 24 hours after seeding, cells were treated with 10 μM VU0364739 or 20 μM VU0359595 in neurobasal media without supplements for an additional 24 hours. Viability was measured as in (A). **D**. Anchorage independent growth of glioma stem cell clone 4302 was assessed using soft-agar colony formation. Growth media was replaced every 2-3 days with 10 μM PLD inhibitor or vehicle. Colonies were allowed to form for 8 weeks. Large colonies were scored after visualization with Crystal Violet. For (C and D), * $p < 0.5$, ** $p < 0.01$, *** $p < 0.005$, *NS* - not significant, unpaired Student's t-test. Error bars - (SEM).

PLD2 is required for Akt activation in GBM cells

After establishing a requirement for PLD2 in glioma cell viability, we wanted to determine the mechanism by which PLD2 regulates survival signaling. The PI3K/Akt pathway is frequently upregulated in cancer and promotes survival by inhibiting apoptotic

processes and by regulating metabolism and nutrient utilization (reviewed in Manning and Cantley, 2007). Additionally, extracellular pathogens are known to engage the Akt pathway upon infection, and bacterial PLD from *N. gonorrhoeae* was demonstrated to interact with and activate human Akt upon infection of human cervical epithelial cells (Edwards and Apicella, 2006). Thus, we hypothesized that human PLD may also interact with Akt. In order to determine if human PLD2 regulates Akt activation in PTEN-null glioma lines, we measured Akt phosphorylation following treatment with PLD inhibitors under various growth conditions. Under the canonical Akt activation sequence, PI3K generates PIP_3, which serves as a membrane recruitment signal for Akt (Franke et al., 1995; James et al., 1996). Membrane-bound Akt is subsequently activated via a phosphorylation dependent mechanism whereby 3-phosphoinositide dependent kinase 1 phosphorylates Akt at threonine 308 in the activation loop and other kinases such as the mTOR complex 2 (mTORC2) phosphorylate Akt in its hydrophobic motif at serine 473 (Alessi et al., 1997; Sarbassov et al., 2005). Cells were treated overnight with PLD inhibitors in either serum-free DMEM, DMEM + 10% FBS, or DMEM followed by stimulation for 10 minutes the following day with 20% FBS. Since the PLD1- and PLD2-preferring inhibitors are chemically unique compounds that have few structural similarities, using either inhibitor individually at concentrations high enough to inhibit both isozymes (Figure 1B) without causing substantial cell death (Figure 2A) allowed us to minimize possible off-target effects associated with an individual compound. Inhibition of PLD in the PTEN-null U87MG (Figure 3A and B) and U118MG (Figure 3C and D) cell lines resulted in decreased levels of activated Akt under serum-depleted conditions as assessed by phosphorylation of threonine 308 and serine 473. Akt phosphorylation was less affected by PLD inhibition when cells were cultured normally or when stimulated with 20% FBS. These results strongly suggest that PLD regulates Akt activation predominantly under stressful, serum-depleted conditions. Additionally, these results

suggest that the PLD inhibitors do not inhibit upstream kinases or Akt directly since growth factor signaling to Akt remains unperturbed. By contrast, PLD inhibitors do not reduce phosphorylated Akt in the non-tumorigenic HEK293 cell line under any condition (Figure 3E and F), suggesting a cell-type specific regulation of Akt by PLD. We next knocked down either PLD1 or PLD2 to dismiss any off-target effects of PLD inhibitors and also to further link a specific PLD isoform to this process. Transfection of U87MG cells with PLD2, but not PLD1, siRNA resulted in a significant decrease in phosphorylated Akt at both threonine 308 and serine 473 (Figure 3G and H). Taken together, the data demonstrate that PLD regulates Akt activation under serum-depleted conditions and that regulation is due to the PLD2 isoform.

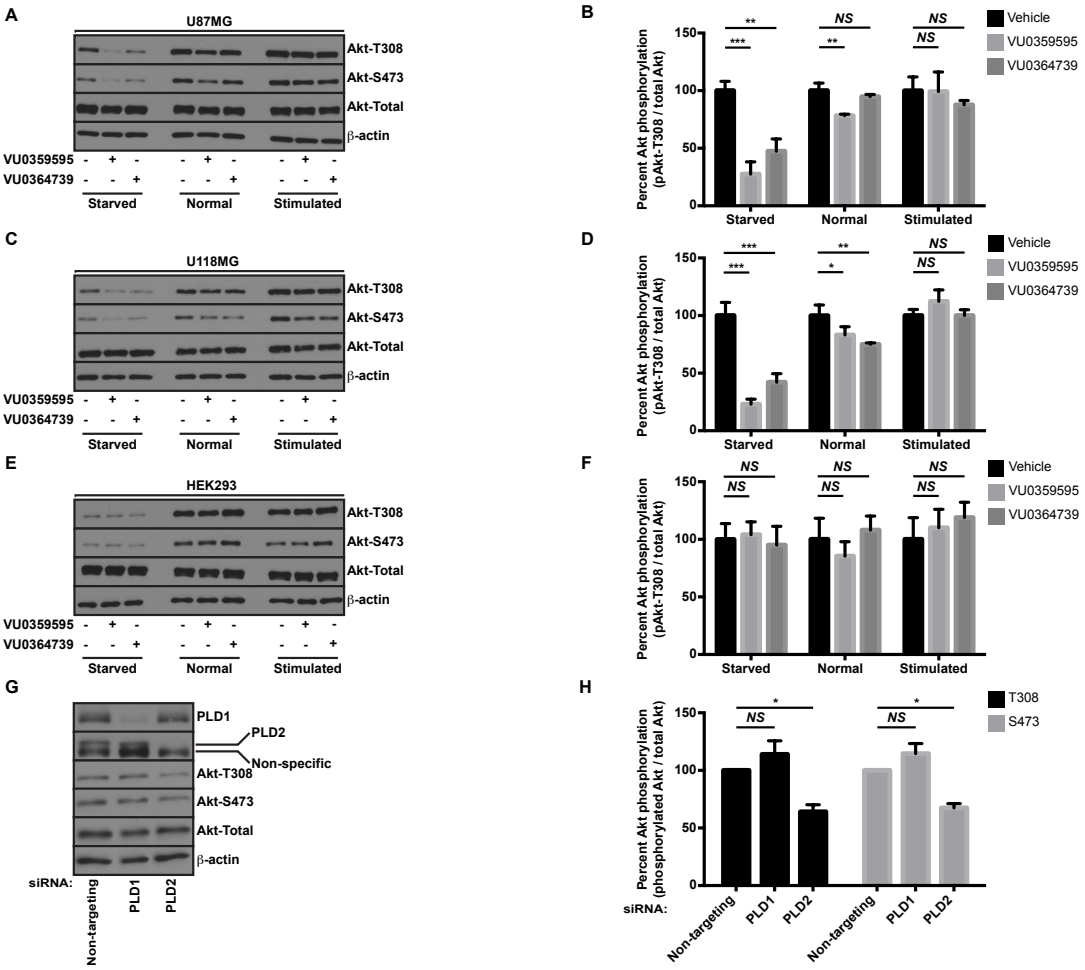

Figure 3. Akt activation requires PLD activity in GBM cells. U87MG (**A** and **B**), U118MG (**C** and **D**), or HEK293 (**E** and **F**) were incubated overnight in the presence of 10 μM VU0359595 or 5 μM VU0364739 in either DMEM ("starved") or DMEM + 10% FBS ("normal"). Another set of cells were starved overnight then stimulated the following day with 20% FBS for 10 minutes ("stimulated") for comparison. Akt activation was assessed by immunoblotting for phosphorylation at T308 and S473. Blots were quantified by calculating the ratio of phospho-Akt at T308 to total Akt using densitometry. S473 was not quantified since no qualitative differences were seen between S473 and T308. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, ANOVA with Dunnett's post-hoc test comparing the PLD inhibitor treatment to vehicle within the growth condition. **G**. U87MG cells were transfected with either PLD1 or PLD2 siRNA for 48 hours prior to overnight serum starvation and immunoblotting. **H**. Quantification of phospho-Akt (T308 and S473) following PLD siRNA treatment in U87MG cells. Data represent the fold change in phospho-Akt relative to the non-targeting siRNA controls and are averages from three independent experiments. * $p < 0.05$, paired Student's t-test. Error bars - SEM. *NS* - not significant.

Phosphatidic acid regulates Akt activation

Since PLD is required for Akt activation following serum-withdrawal in GBM cells, we next determined whether human PLD forms a protein complex with Akt, as was suggested with PLD from *N. gonorrhoeae* (Edwards and Apicella, 2006). Due to the lack of available antibodies suitable for immunoprecipitating endogenous PLD proteins, FLAG-tagged PLD1 or PLD2 was transfected and IP'ed from U87MG cells to probe for co-IP of endogenous Akt. When PLD complexes were probed for binding of Akt, co-IP was detected in PLD2 but not PLD1 complexes (Figure 4A). Since overexpression of proteins is prone to artifacts, we next IP'ed endogenous Akt from U87MG cells to probe for co-IP of endogenous PLD2. Under conditions of native levels of protein expression, we detected co-IP of PLD2 with Akt, confirming the formation of a protein complex between PLD2 and Akt in U87MG cells (Figure 4B). In order to determine if PLD2 interacts directly with Akt, we expressed and purified recombinant Akt1 and PLD2 from Sf21 insect cells (Figure 4C) and performed *in vitro* binding assays using strep-tactin affinity resin to capture protein complexes. Purified Akt1 bound PLD2 in the absence of other proteins *in vitro*, suggesting that the PLD2 and Akt1 form a direct interaction (Figure 4D).

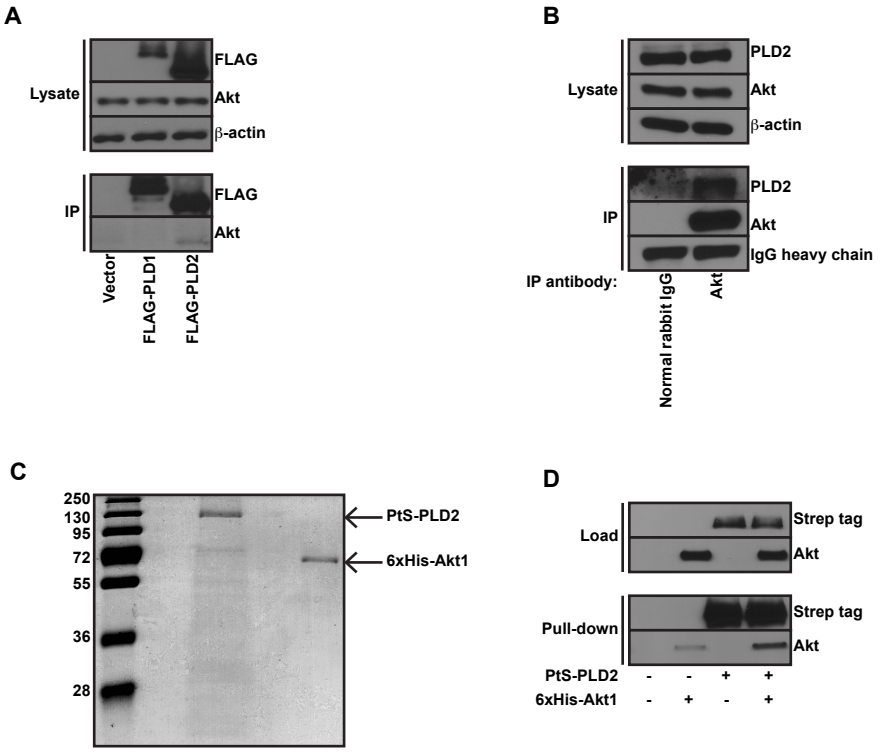

Figure 4. Akt and PLD2 form a direct protein complex. **A**. U87MG cells were transfected with vector or FLAG-tagged PLD1 or PLD2 for 48 hours. FLAG-PLD1 or PLD2 was immunoprecipitated and binding of endogenous Akt was assessed by immunoblotting FLAG-PLD complexes for Akt. **B**. U87MG cells were lysed and endogenous Akt immunoprecipitated overnight using a pan-specific Akt antibody. Non-specific proteins were immunoprecipitated using normal rabbit IgG. Note, approximately 0.5% of the material used for IP was loaded into the lysate lanes for (A) and (B). **C**. Coomassie brilliant blue stained gel of PLD2 and Akt to demonstrate protein purity. Numbers indicate molecular weights in kDa. **D**. Purified Akt was incubated with either PLD2 or PtS tag for 2 hours and complexes captured using affinity resin. Bound Akt was determined by immunoblotting for Akt to demonstrate a direct protein-protein interaction between PLD2 and Akt.

In order to gain insight into the nature of the PLD2-Akt interaction, we sought to determine which domains of each protein were responsible for mediating the interaction. Akt has three main domains including a PH domain, a kinase domain, and hydrophobic motif (HM). We created truncation mutants based on published crystal structures; the Akt PH domain construct encoded amino acids 1-123 (Thomas et al., 2002) and the Akt kinase domain encoded amino acids 146-480 (Akt.d145) (Yang et al., 2002). Additionally, we created two PLD2 truncation mutants encoding the PX and PH domains

(amino acids 1-308) and a C-terminal fragment (amino acids 309-933), known to retain catalytic activity (Sung et al., 1999a). Full-length proteins and deletion mutations were ligated into vectors to express N-terminal epitope tags including PtS, maltose-binding protein (MBP), glutathione S-transferase (GST), or 6xHis to facilitate protein purification and *in vitro* binding assays. In order to assess Akt fragment binding to PLD2, we performed saturation binding assays by incubating 25 nM PLD2 or PLD2 fragment with increasing concentrations of full-length Akt, Akt-PH, or Akt.d145. PLD2 fragments were captured using affinity resin. We assessed specific binding of Akt to PLD2 by subtracting any Akt bound to PtS or MBP tag control proteins. Full-length PLD2 specifically bound full-length Akt and Akt.d145 but did not bind the Akt PH domain (Figure 5). On the other hand, PLD2.d308 did not specifically bind any of the Akt constructs (Figure 6). The PLD2-PXPH construct specifically bound full-length Akt and Akt.d145, similarly to full-length PLD2, suggesting that the PLD2-PXPH domain interacts with the kinase domain and hydrophobic motif of Akt (Figure 7).

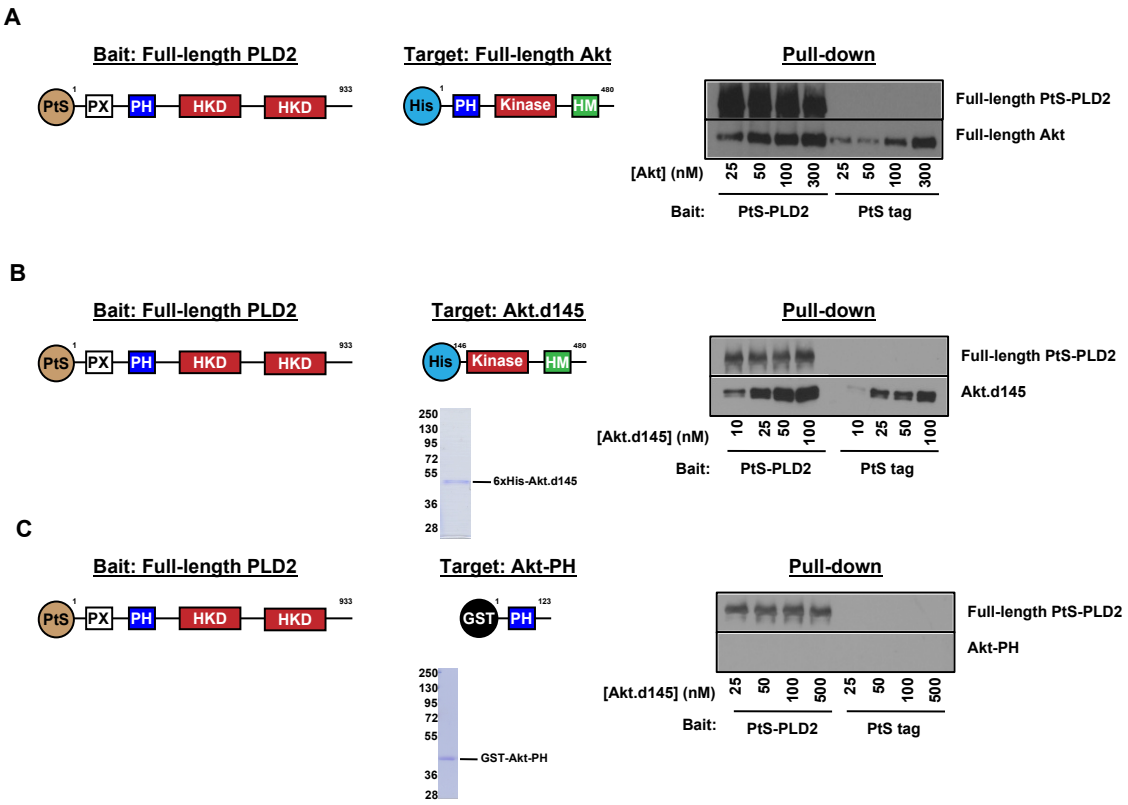

Figure 5. Saturation binding analysis of Akt fragments to full-length PLD2. Full-length PtS-PLD2 or PtS tag (25nM) was incubated with the indicated concentration of Akt fragment for 2 hours before incubating complexes for 2 hours with strep-tactin affinity resin. Complexes were washed and eluted by boiling in 2x SDS-PAGE loading buffer. Specific binding is assessed as increased levels of Akt protein present in the PtS-PLD2 samples versus PtS tag controls. Coomassie stained gels of Akt.d145 and Akt-PH are shown to demonstrate purity. Numbers are molecular weights in kDa.

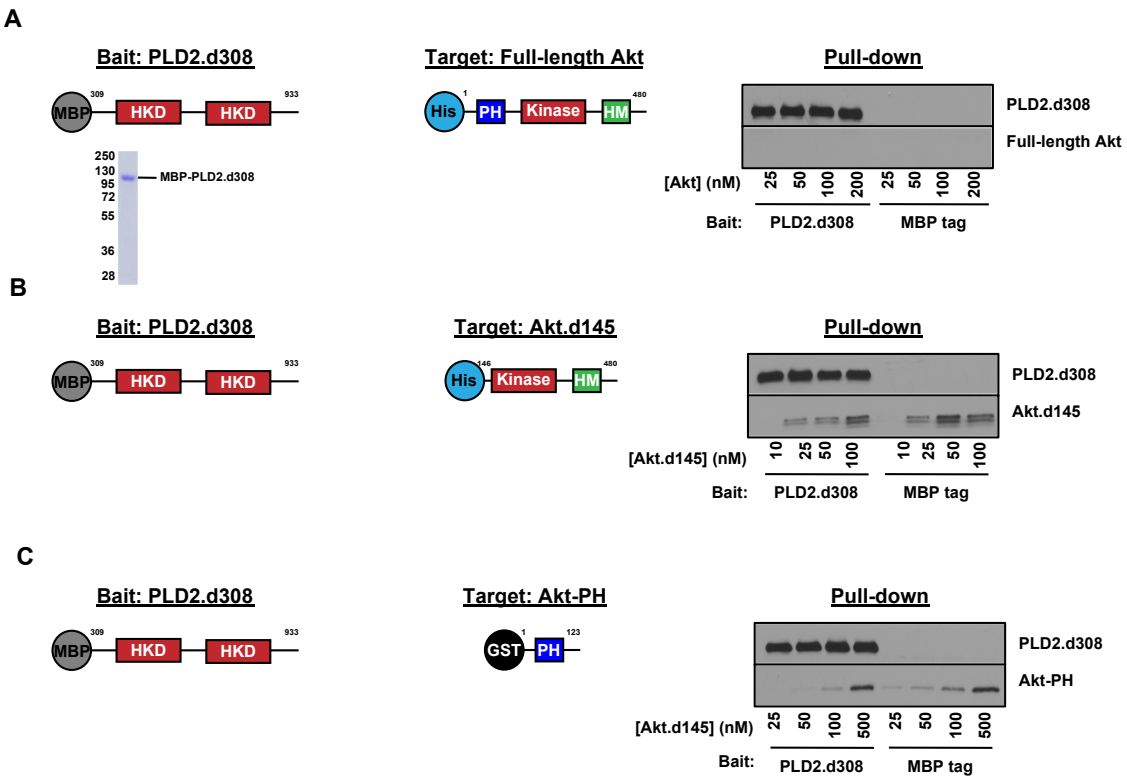

Figure 6. Saturation binding analysis of Akt fragments to PLD.d308. MBP-tagged PLD2.d308 or MBP tag (25nM) was incubated with the indicated concentration of Akt fragment for 2 hours before incubating complexes for 2 hours with amylose resin to capture MBP proteins. Complexes were washed and eluted by boiling in 2x SDS-PAGE loading buffer. Specific binding is assessed as increased levels of Akt protein present in the PLD2.d308 samples versus MBP tag controls. Coomassie stained gel of PLD2.d308 is shown to demonstrate purity. Numbers are molecular weights in kDa.

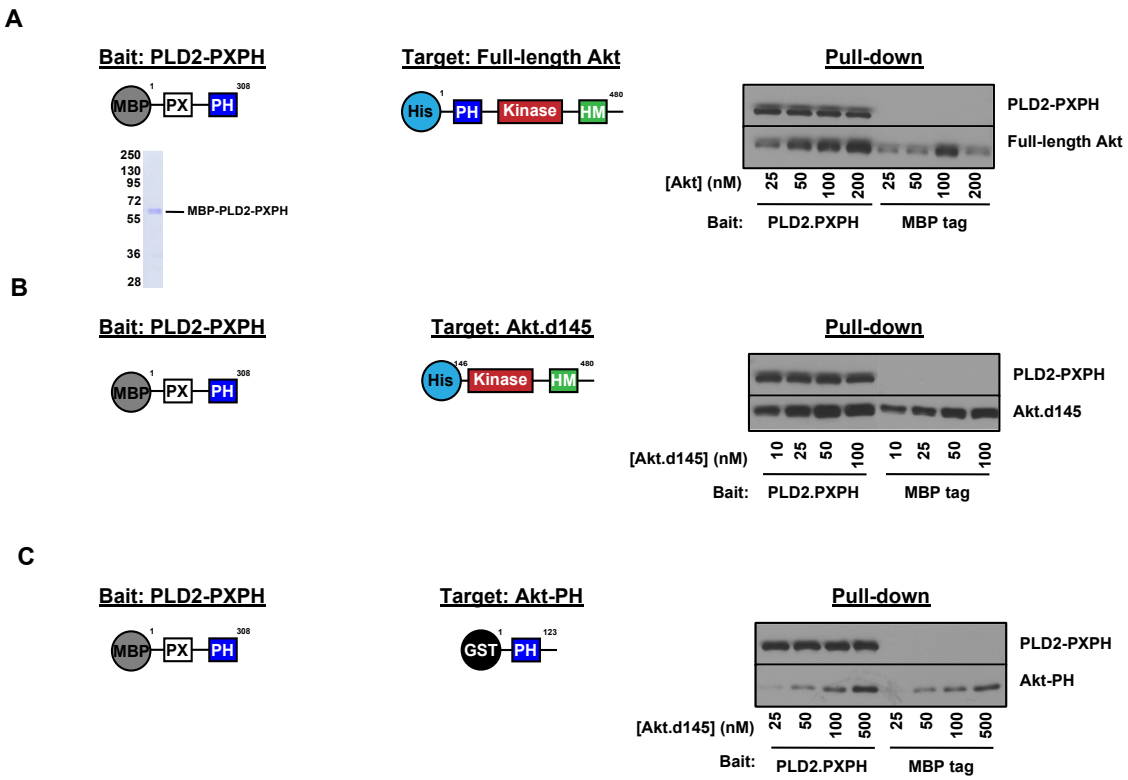

Figure 7. Saturation binding analysis of Akt fragments to PLD-PXPH. MBP-tagged PLD2-PXPH or MBP tag (25nM) was incubated with the indicated concentration of Akt fragment for 2 hours before incubating complexes for 2 hours with amylose resin to capture MBP proteins. Complexes were washed and eluted by boiling in 2x SDS-PAGE loading buffer. Specific binding is assessed as increased levels of Akt protein present in the PLD2-PXPH samples versus MBP tag controls. Coomassie stained gel of PLD2-PXPH is shown to demonstrate purity. Numbers are molecular weights in kDa.

After establishing that PLD2 interacts with the Akt C-terminus through its PX and PH domains, we next determined whether the protein interaction regulates enzymatic activity. First, we performed *in vitro* choline release PLD activity assays in the presence or absence of Akt protein. We saw no changes in PLD activity by including Akt protein in the reaction mixture (data not shown). However, full-length PLD2 stimulated Akt kinase activity towards a substrate peptide around two-fold (Figure 8A). Likewise, the PLD2-PXPH domain also stimulates Akt kinase in vitro consistent with observation that the PLD2-PXPH domains interact with the Akt kinase domain (Figure 8B). The observation that PLD2 stimulates Akt kinase activity in the absence of substrate or product lipids suggests that PLD regulates Akt kinase activity in a lipase-independent manner.

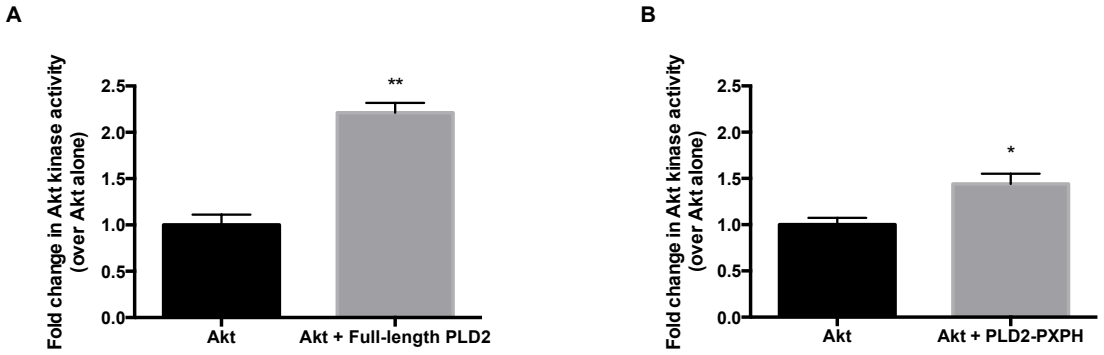

Figure 8. Full-length PLD2 and the PX/PH domains of PLD2 stimulate Akt kinase activity *in vitro*. 50 nM of purified Akt kinase was incubated with or without 50 nM (**A**) PtS-PLD2 or (**B**) PLD2-PXPH protein in the presence of 100 μM ATP and a GST-GSK3 Akt substrate peptide. Kinase reactions were terminated by boiling samples in 2x SDS-PAGE loading buffer. Akt kinase activity was determined by immunoblotting with a phospho-GSK3 antibody. Note; no lipids were included in the assay. Data is presented as the fold change in Akt kinase activity due to the presence of PLD proteins over the amount of Akt kinase activity in the absence of PLD proteins. * $p < 0.05$, ** $p < 0.01$, unpaired Student's t-test.

In order to determine if PLD inhibitors disrupted the PLD2-Akt complex, we performed in vitro binding assays in the presence of VU0359595 and VU0364739 or the allosteric pan-Akt inhibitor MK2206 (Hirai et al., 2010). PLD inhibitors did not disrupt the

PLD2-Akt complex, suggesting that PLD inhibitors decreased Akt activation independently of the PLD2-Akt interaction (Figure 9). Since PLD inhibitors did not disrupt the PLD2-Akt protein complex, we investigated a potential regulation of Akt by PtdOH, the catalytic product of PLD. To confirm that the decrease in Akt phosphorylation following PLD inhibitor treatment or siRNA knockdown was due to the decrease in PtdOH production, we attempted to rescue Akt phosphorylation by co-treatment of U87MG cells with PLD inhibitors and exogenously added PtdOH. Our laboratory recently published a detailed lipidomic characterization of PtdOH species generated by PLD in an astrocytoma cell line (Scott et al., 2013). Based upon this analysis and others, we attempted to rescue Akt phosphorylation using 36:2 PtdOH, a species generated by PLD (Scott et al., 2013). We observed complete rescue of Akt phosphorylation with exogenously added PtdOH (Figure 10A and B), suggesting that decreased Akt phosphorylation following PLD inhibitor treatment was due to decreased production of PtdOH by PLD.

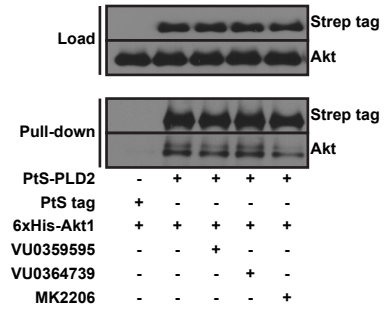

Figure 9. PLD inhibitors do not disrupt the PLD2-Akt complex. 50 nM Akt was incubated with either 25 nM PLD2 or PtS tag for 2 hours and complexes captured using strep-tactin affinity resin. Where indicated, 10 μM VU0359595, VU0364739, or Akt inhibitor MK2206 was included in the reaction mixture. Akt binding was assessed by immunoblotting strep-tactin-bound PLD2 for the presence of Akt.

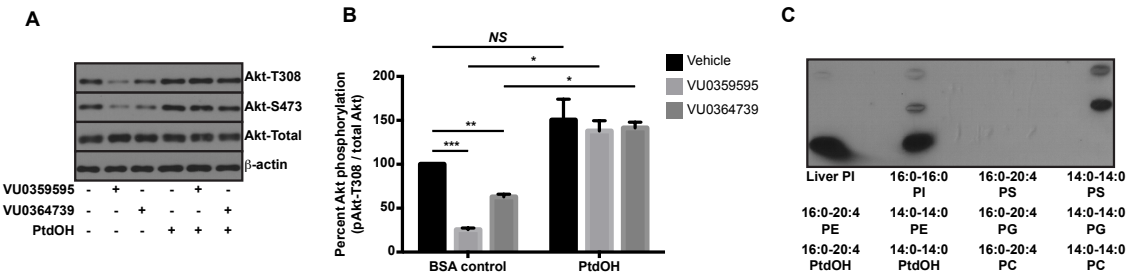

Figure 10. PtdOH increases Akt activation. **A**. U87MG cells were treated overnight with 10 μM VU0359595 or 5 μM VU0364739 in DMEM containing 0.25 mg/ml fatty-acid free BSA. Where indicated, cells were co-treated with 1 mM PtdOH or BSA control and Akt phosphorylation was assessed by immunoblotting T308 and S473. **B**. Quantification of PtdOH rescue of Akt phosphorylation following PLD inhibitor treatment. Fold changes in phospho-Akt (T308) were determined relative to the vehicle treated, BSA control. Data were analyzed by repeated measures ANOVA across all conditions and post-hoc paired t-tests in indicated conditions. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **C**. 5 nM recombinant Akt was incubated overnight with nitrocellulose membranes containing 2 μg of the indicated lipid. Bound Akt was measured using an Akt specific antibody. Phospholipids are denoted XX;Y, where XX refers to the number of carbon atoms in the acyl chain and Y indicates the degree of unsaturation.

Several studies have recently suggested that Akt binds other phospholipids in addition to phosphoinositides including phosphatidylserine (Huang et al., 2011) and PtdOH (Mahajan et al., 2010). To compare the relative affinity of Akt for various phospholipids, we measured Akt binding using a commercially available protein-lipid overlay assay (Dowler et al., 2002) in which phospholipids are spotted onto nitrocellulose and binding of recombinant protein is detected immunologically. In agreement with other studies, recombinant Akt bound PtdOH and with higher affinity than other phospholipids (Figure 10C).

PtdOH enhances Akt binding to PIP_3 and subsequent membrane recruitment

In order to understand the mechanism by which PtdOH regulates Akt activation, we first determined whether PtdOH and PIP_3 share a binding site on Akt. Recombinant Akt was incubated with lipid vesicles composed of PC alone or PC with PtdOH. Akt

binding to PtdOH and PIP_3 was then assessed using a protein-lipid overlay assay. When pre-incubated with vesicles containing PtdOH, binding of Akt to PtdOH on nitrocellulose was diminished, and this condition served as an internal control for the experiment (Figure 11A). Intriguingly, binding of Akt to PIP_3 was strongly enhanced by pre-incubation with PtdOH-containing vesicles (Figure 11A). Since PIP_3 is known to bind the Akt PH domain based on published crystal structures (Thomas et al., 2002), we investigated whether the PH domain also mediates the interaction with PtdOH. GST-Akt PH domain fusion proteins were purified from E. coli and lipid binding was again assessed using a protein-lipid overlay. We purified the wild-type (WT) Akt PH domain and an Akt PH domain mutant deficient in PIP_3 binding, R25C (Thomas et al., 2002), to determine if perturbing PIP_3 binding would also alter PtdOH binding. The WT PH domain of Akt was sufficient to bind PtdOH and disruption of PIP_3 binding with the R25C mutant had no effect on PtdOH binding (Figure 11B). These results suggest that PtdOH binds a distinct site on the PH domain of Akt and that the binding of PtdOH acts cooperatively to increase the affinity of Akt for PIP_3.

Since PIP_3 recruits Akt to membranes (Bellacosa et al., 1991; Franke et al., 1995) and PtdOH increased Akt binding to PIP_3, we investigated the possibility that PLD-generated PtdOH regulates membrane localization of Akt. Membranes from serum-starved U87MG cells treated with vehicle or PLD inhibitors were prepared by flotation through a discontinuous iodixanol gradient. Under control conditions, Akt was present in both cytosolic and membrane fractions and as we hypothesized, inhibition of PLD decreased the levels of both total and phosphorylated Akt in the membrane fraction (Figure 11C). Akt membrane recruitment was less sensitive to PLD inhibition in the presence of serum and Akt was not detected in the membrane fractions of serum-starved HEK293 cells except under conditions where film was extremely overexposed (data not shown), consistent with constitutive activation of Akt in PTEN-null GBM cells.

To confirm that PLD inhibitors decreased Akt membrane localization in a PtdOH dependent manner, we co-treated U87MG cells with PLD inhibitors and the PLD product PtdOH before preparing membranes. Interestingly, co-treatment of cells with PtdOH not only rescued Akt membrane localization but PtdOH treatment resulted in a dramatic relocalization of cytosolic Akt to the membrane fraction (Figure 11D). These data implicate PLD-generated PtdOH as a crucial mediator of Akt-membrane recruitment in GBM cells.

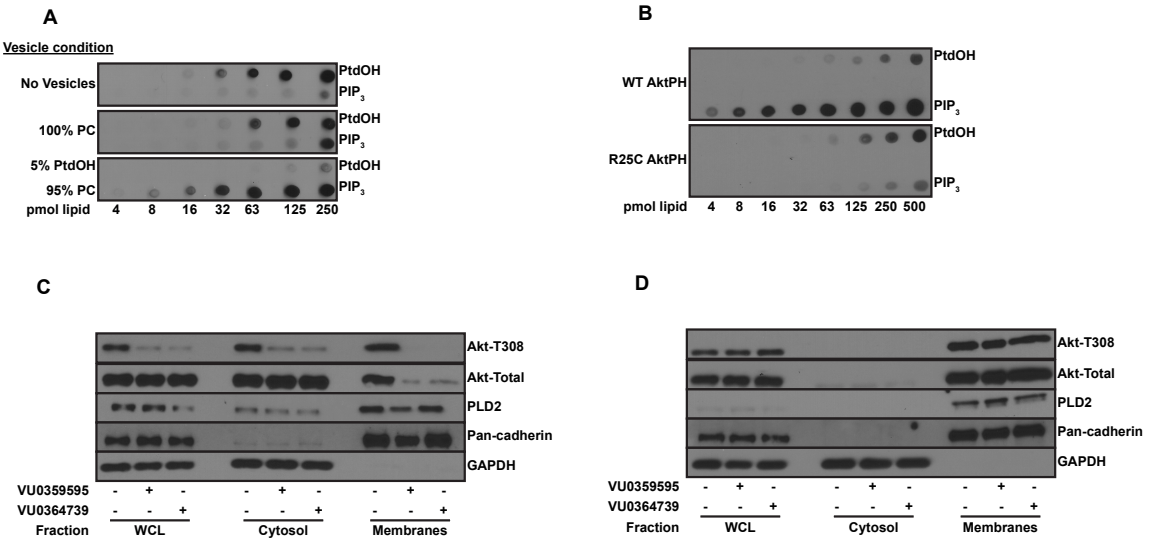

Figure 11. Akt recruitment to membranes is enhanced by binding to PtdOH. **A**. Recombinant Akt (3 nM) was incubated with 200 μM bulk lipid vesicles for 1 hour then the Akt-vesicle mixtures were incubated overnight with nitrocellulose spotted with PtdOH or PIP_3. Bound Akt was determined using an Akt specific antibody. **B**. Protein-lipid overlay measuring wild-type or R25C PIP_3 binding deficient mutant Akt PH domain (3 nM) binding to PtdOH or PIP_3. **C** and **D**. U87MG cells were treated for 6 hours with 10 μM PLD inhibitor in the absence (C) or presence (D) of 1 mM 36:2 PtdOH in DMEM + 0.25 mg/ml BSA and separated into cytosol and membranes for immunoblotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pan-cadherin were used as cytosolic and membrane markers, respectively. Error bars - SEM. WCL – whole cell lysate, *NS* – not significant.

PLD2 inhibition induces autophagy-dependent cell death

After establishing a requirement for PLD2-generated PtdOH in the activation of Akt in U87MG cells, we next determined the mechanism of cell death following inhibition of the PLD2-Akt pathway by first measuring markers for apoptosis in U87MG cells. PLD inhibition only modestly induced caspase-3/7 cleavage relative to a well-characterized apoptotic stimulus, staurosporine (Bertrand et al., 1994) and treatment of U87MG cells with a pan-caspase inhibitor failed to rescue PLD-inhibitor induced cell death (Figure 12). Together, these results suggest that PLD inhibition predominantly leads to non-apoptotic cell death in this GBM line.

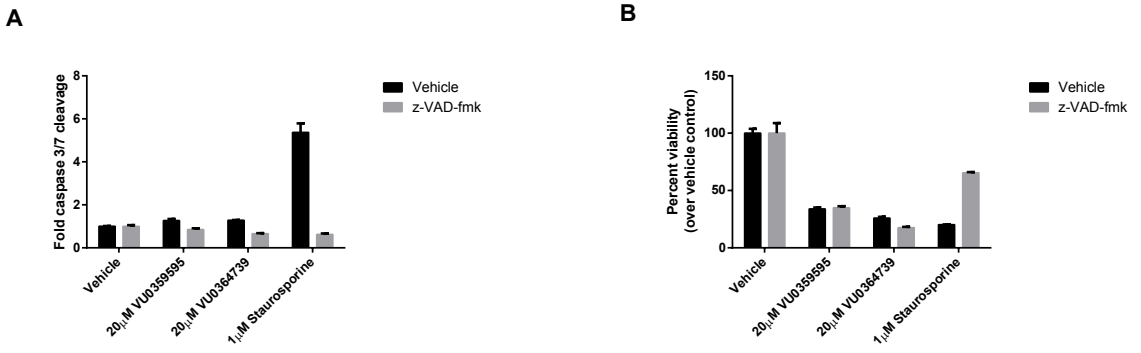

Figure 12. PLD inhibition does not induce apoptotic cell death. **A**. U87MG cells treated overnight with PLD inhibitor in the presence of 50 μM z-VAD-fmk, a pan caspase inhibitor. 24 hours after treatment, caspase 3/7 cleavage was measured using the luminometric Caspase-Glo 3/7 kit from Roche. **B**. Viability was measured using the WST-1 reagent. Staurosporine was included as a positive apoptotic control. Error bars - SEM.

In addition to apoptosis, cells undergo another type of programmed cell death requiring autophagy (Tsujimoto and Shimizu, 2005), a process known to be stimulated by nutrient or growth factor deprivation (Kroemer et al., 2010). Autophagy is a multistep process involving formation of double-membrane autophagosomes that engulf cytosolic components and deliver cargo to lysosomes for digestion and nutrient recycling

(Ravikumar et al., 2009). In order to determine if autophagy was perturbed following PLD or Akt inhibitor (MK2206) (Hirai et al., 2010) treatment, we measured the expression levels of the well-characterized autophagy markers microtubule-associated protein 1A/1B-light chain 3 (LC3) and p62. Autophagosome number is frequently assessed by measuring conversion of cytosolic LC3-I to the membrane-associated, lipidated LC3-II which is readily measured as a faster migrating species of LC3 during SDS-PAGE (Kabeya et al., 2000). The other marker, p62, is an LC3 and ubiquitin-binding protein involved in the regulation of protein aggregates and is degraded by autophagy (Komatsu et al., 2007). Induction of autophagy and successful degradation of autophagosomes would thus be accompanied by a decrease in p62 levels. Overnight treatment of U87MG cells with PLD or Akt inhibitors robustly induced LC3-II conversion and also increased p62 levels (Figure 13A and B). These results suggest an increased number of autophagosomes resulting from a deficiency in autophagosome turnover as is often observed under conditions where autophagy is defective (Wang et al., 2006). In the presence of PtdOH, PLD inhibitors failed to increase autophagy markers, which further validates the specificity of our inhibitors (Figure 13C, D, and E). LC3-II and p62 levels also increased in other glioma cell lines including the CD133+ glioma stem cells (Figure 13F) and U118MG cells (data not shown) following PLD inhibitor treatment.

To determine if the effects on autophagy following PLD inhibition were cell-type specific, we compared LC3/p62 levels between U87MG cells and HEK293 cells. PLD and Akt inhibitors increased LC3-II conversion in both cell types (Figure 13G). However, the levels of LC3 and p62 under basal conditions were much higher in the U87MG cells, suggesting that glioma cells utilize autophagy more so than other cell types, rendering them particularly sensitive to compounds that perturb autophagy.

To confirm that U87MG cells were undergoing autophagy-dependent cell death, we measured the ability of PLD inhibitors to decrease viability when machinery required

for autophagosome formation was perturbed by siRNA knockdown. Autophagy-related protein 7 (Atg7) is a ubiquitination E1-like enzyme required for autophagosome formation (Komatsu et al., 2005). Knockdown of Atg7 significantly increased viability (Figure 13H) and decreased LC3-II conversion (Figure 13I) following PLD inhibition in U87MG cells. These results strongly suggest that GBM cell death resulting from PLD inhibition is predominantly through an autophagy-dependent mechanism.

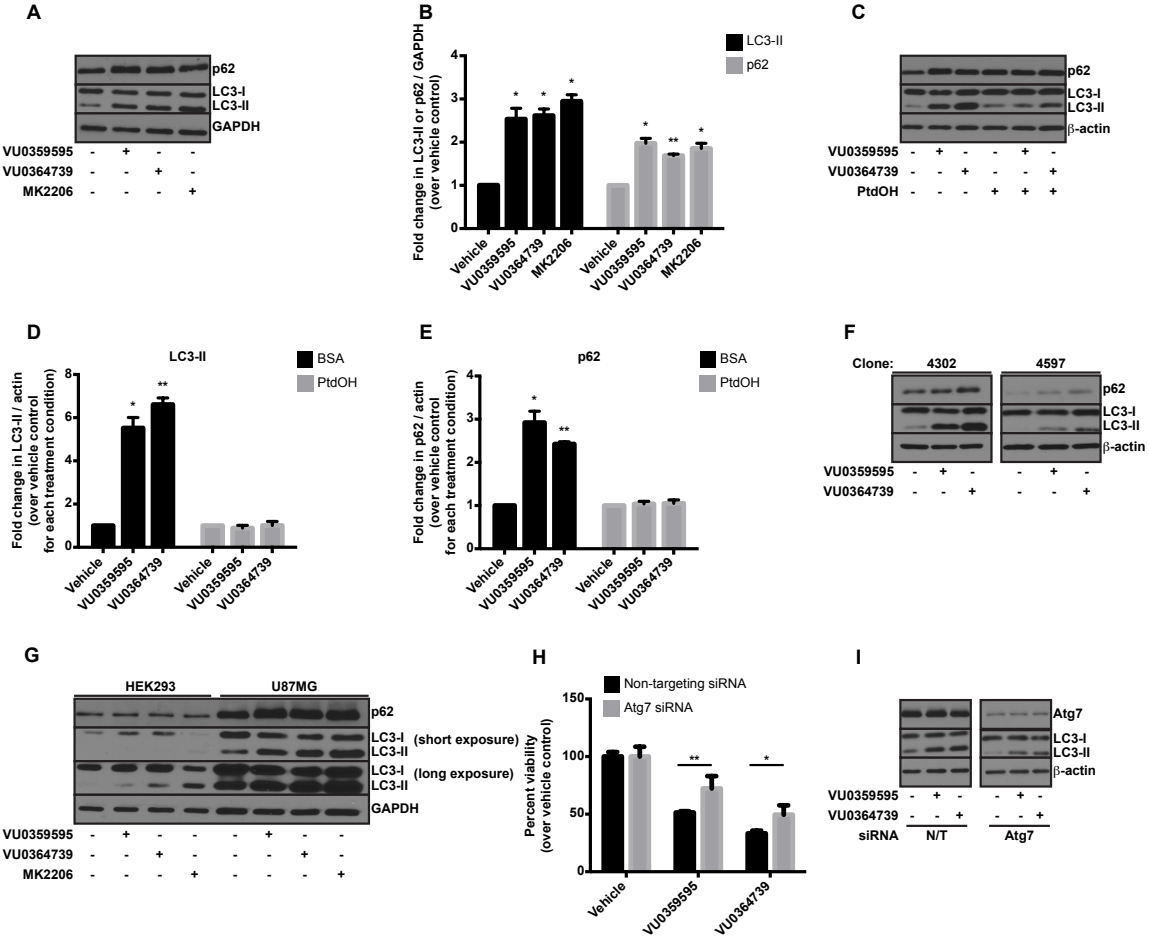

Figure 13. PLD inhibitors induce autophagy dependent cell death in GBM. **A**. Immunoblot of autophagy markers from U87MG cells treated overnight with 10 μM VU0359595, 5 μM VU0364739, or 10 μM MK2206 in serum-free DMEM. **B**. Quantification of LC3-II and p62 increase in U87MG cells following PLD or Akt inhibition. Fold change in immunoreactivity relative to vehicle control. * $p < 0.05$, ** $p < 0.01$, ANOVA with Dunnett's post-hoc test comparing inhibitor treatment to vehicle control. **C**. Immunoblot of autophagy markers from U87MG cells treated overnight with PLD inhibitors with or without 1 mM 36:2 PtdOH in DMEM + 0.25 mg/ml BSA. **D** and **E**. Quantification of LC3-II and p62 from (C). * $p < 0.05$, ** $p < 0.01$, ANOVA with Dunnett's post-hoc test comparing inhibitor treatment to vehicle control within the PtdOH treatment conditions. **F**. Immunoblot of autophagy markers from CD133+ glioma stem cells treated overnight with 10 μM VU0359595 or 5 μM VU0364739 in neurobasal media without growth supplements. **G**. Comparison of autophagy marker expression in U87MG or HEK293-TREx cells treated overnight with 10 μM VU0359595, 5 μM VU0364739, or 10 μM MK2206 in serum-free DMEM. **H**. Viability assay from U87MG cells treated with siRNA targeting Atg7. Cells were treated overnight with PLD or Akt inhibitors in serum-free DMEM before measuring viability with the WST-1 reagent. * $p < 0.05$, ** $p < 0.01$, two-way ANOVA with post-hoc Sidak multiple comparison test on Atg7 siRNA effect within inhibitor treatment groups. **I**. Immunoblot of LC3 and Atg7 for experiment in panel (H). Error bars - SEM. N/T – non-targeting siRNA.

PLD and Akt inhibition reduces autophagic flux

The increased conversion of LC3-II and increased expression of p62 after inhibitor treatments in glioma cells suggests that autophagic flux requires PLD and Akt activity. To conclusively determine that flux, rather than autophagosome initiation, is regulated by PLD, we measured LC3-II conversion in the presence of the lysosomal proton pump inhibitor bafilomycin A1, which prevents autophagosome fusion to lysosomes and inhibits degradation of autophagosomes. Thus, bafilomycin A1 is commonly used to discriminate the effects of a compound on autophagy initiation versus flux by assessing LC3-II levels in the presence of a test compound after clamping degradation of autophagosomes (Yamamoto et al., 1998a). Bafilomycin A1, PLD, and Akt inhibitor treatment increased LC3-II levels relative to vehicle control (Figure 14A and B). However, no additional accumulation of LC3-II was measured when PLD or Akt inhibitors were added in the presence of bafilomycin A1, confirming that PLD and Akt were controlling degradation of autophagosomes.

To further demonstrate decreased degradation of autophagosomes following PLD2/Akt inhibition, we generated a stable U87MG cell line to express a tandem-fluorescent LC3 reporter (tf-LC3) used to assess autophagosome maturation (Kimura et al., 2007). This reporter system consists of a red fluorescent protein (RFP) and green fluorescent protein (GFP) fused to LC3. As autophagosome numbers increase, either due to increased autophagy initiation or decreased degradation, fluorescence intensity increases as LC3 clusters on autophagosome membranes. Unlike RFP, GFP is quenched by low pH and LC3 present in lysosomes should predominantly emit an RFP signal. Under situations where autophagosome degradation is perturbed, the GFP and RFP signals co-localize since autophagosomes do not fuse to acidic lysosomes. The tf-LC3 U87MG cells were treated with PLD or Akt inhibitors overnight, fixed, and imaged for GFP and RFP signals. Under vehicle treated conditions, numbers of LC3 puncta

were low and predominantly visualized with the pH-insensitive RFP tag, indicative of functional autophagy. However, PLD or Akt inhibitor treatments induced a robust relocalization of cytosolic LC3-I to large fluorescent puncta and when merged, the GFP/RFP signals highly co-localized, indicating a perturbation in the ability of the cell to effectively degrade and process autophagosomes (Figure 14C).

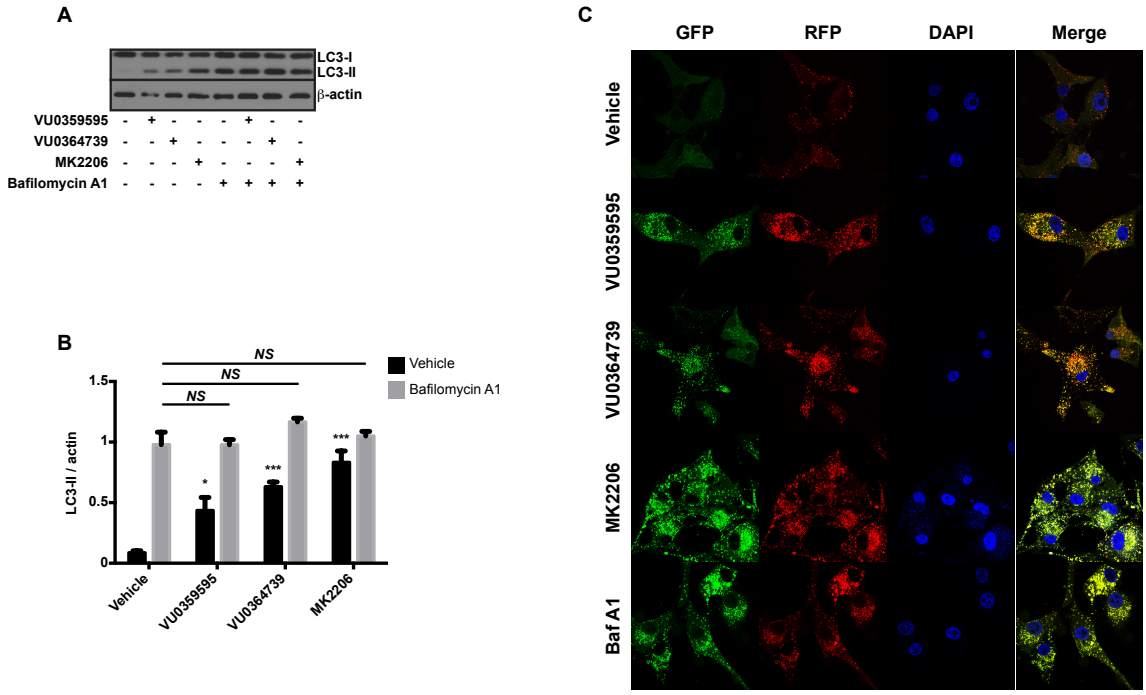

Figure 14. PLD inhibition decreases autophagic flux. **A**. Immunoblot of LC3 from U87MG cells treated with 10 nM bafilomycin A1 for 30 minutes followed by treatment with 10 μM VU0359595, 5 μM VU0364739, or 10 μM MK2206 for 6 hours in serum-free DMEM before cell harvest. **B**. Quantification of LC3-II conversion in the presence of bafilomycin A1 and PLD/Akt inhibitors. Data are presented as the ratio of band intensity for LC3-II relative to Actin. * $p < 0.05$, *** $p < 0.005$, two-way ANOVA with Tukey's post-hoc test on PLD/Akt inhibitor effects versus vehicle control, *NS* – no significant impact of PLD inhibitors in conditions with bafilomycin A1. **C**. Representative images of U87MG stable cells expressing a GFP/RFP-LC3 tandem-fluorescent tag. Cells were treated overnight in serum-free DMEM with 10 μM VU0359595, 5 μM VU0364739, 10 μM MK2206, or 10 nM Bafilomycin A1 then fixed and imaged using confocal microscopy. Error bars - SEM.

After establishing that PLD and Akt promote autophagic flux, we sought to understand the molecular mechanism. The mTOR pathway suppresses autophagy under nutrient rich conditions and PLD has been implicated as an upstream positive regulator of mTOR (reviewed in Foster, 2009). Although we measured diminution of mTOR activity with Akt inhibition, we measured little to no change in mTOR effector phosphorylation status with PLD inhibition (Figure 15A), suggesting the mTOR pathway was not mediating the effects of PLD inhibitors on autophagy and also suggesting that PLD2 and mTOR signaling are uncoupled in the U87MG cell line. Since mTOR regulation did not explain the effects of PLD inhibition on autophagy, we investigated other Akt substrates. Recently, Akt was shown to phosphorylate beclin1 and promote autophagy (Wang et al., 2012). Beclin1 is a component of the core autophagy complex (Liang et al., 1999) and exists in multiple protein complexes during progressive stages of autophagy (Kihara, 2001). Autophagosome maturation and subsequent degradation is, in part, regulated by the interaction of beclin1 with RUN-domain cysteine rich domain containing, Beclin1 interacting protein (rubicon) (Matsunaga et al., 2009; Zhong et al., 2009), which is believed to negatively impact autophagosome maturation. We hypothesized that the phosphorylation of beclin1 by Akt might inhibit the interaction with rubicon and either PLD2 or Akt inhibition would thereby enhance the interaction. As expected, The PLD2 inhibitor VU0364739 and Akt inhibitor MK2206 increased the amount of rubicon that co-immunoprecipitated with beclin1 from U87MG cells (Figure 15B and C). To address whether the interaction of rubicon with beclin1 was mediated by Akt phosphorylation, we mutated the two putative Akt phosphorylation residues on beclin1, serine 234 and serine 295 (Wang et al., 2012), and assessed rubicon binding. Previous studies identified serine 295 as the predominant Akt phosphorylation site on beclin1 (Wang et al., 2012). Alanine mutation of serine 295, but not 234, increased rubicon binding to beclin1 compared to wild type controls (Figure 15B). PLD and Akt

inhibition failed to increase binding of rubicon to the S295A mutant of Beclin1, supporting the model that Akt activity enhances autophagic flux by preventing binding of Rubicon to Beclin1 (Figure 15B and C).

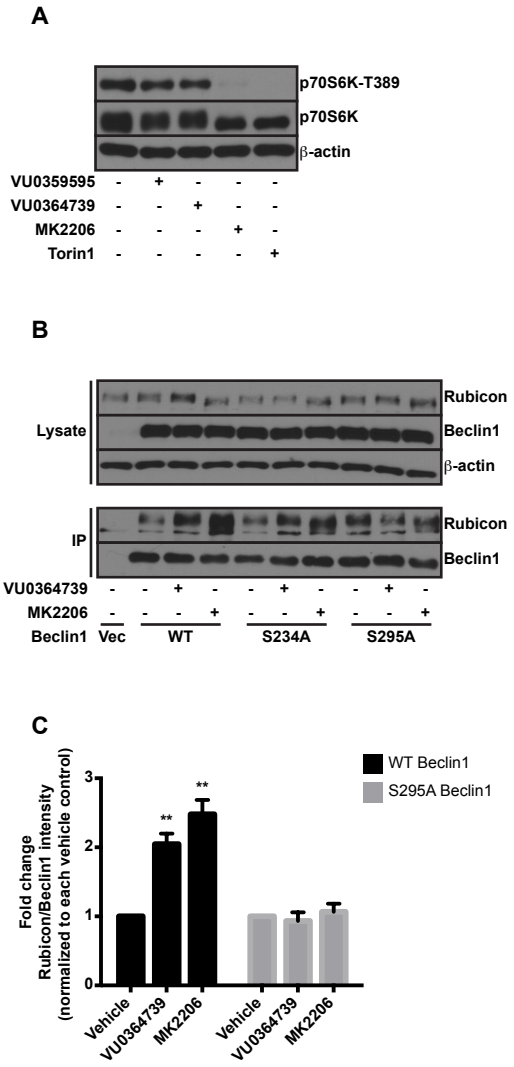

Figure 15. PLD and Akt promote autophagic flux by dissociating rubicon from beclin1. **A**. U87MG cells were treated overnight with 10 μM VU0359595, 5 μM VU0364739, 10 μM MK2206, or 1 μM mTOR inhibitor Torin1. Cells were immunoblotted for total and phosphorylated p70S6K1. **B**. U87MG cells were transfected with HA-tagged wild type or mutant beclin1 for 48 hours. Cells were treated with 10 μM VU0364739 or MK2206 for 6 hours in serum-free DMEM prior to cell harvest and immunoprecipitation of HA-beclin1 with a HA-antibody. Immunoprecipitates were probed for co-IP of endogenous rubicon. **C**. Quantification of the increased binding of rubicon to beclin1 following PLD or Akt inhibition. Band intensities of rubicon and beclin1 were determined and the ratio of rubicon to beclin1 was calculated for each sample. Fold changes in this ratio were calculated by comparing inhibitor conditions to the vehicle treated conditions within the wild-type or S295A beclin1 groups (n=4). ** $p < 0.01$, ANOVA with Dunnett's post-hoc test. Error bars = SEM.

Functional rescue of Akt restores autophagic flux and viability in glioma cells following PLD inhibition

In order to confirm that the effects on autophagy and cell death following PLD inhibition in glioma cells were due to the regulation of Akt by PLD2, we developed a stable U87MG cell line expressing a constitutively active form of Akt under the transcriptional control of the tetracycline repressor protein. This Akt construct contains the myristoylation sequence from Src kinase (Kohn et al., 1996) and is constitutively membrane associated and active. Myr-Akt functions in dominant-positive manner and is the most widely used construct for assessing the cellular effects of constitutive Akt activation (Manning and Cantley, 2007). If PtdOH serves to enhance membrane docking of Akt then PLD inhibition should not decrease phosphorylation of myristoylated Akt (myrAkt1) since this construct bypasses lipid recruitment signals for membrane association. As expected, PLD inhibitors failed to reduce levels of phosphorylated Akt in myrAkt1 U87MG cells (Figure 16A). Unlike PLD inhibitors, Torin1, an ATP-site mTOR inhibitor (Thoreen et al., 2009), decreased phosphorylation of myrAkt1, demonstrating that mTORC2 activity is still required for myrAkt1 phosphorylation and that inhibition of PLD activity does not decrease mTORC2 activity in this cell line (Figure 16B). This result is consistent with PLD regulating Akt by enhancing membrane recruitment rather than regulating kinases or phosphatases that modulate phosphorylation of threonine 308 and serine 473.

Since phosphorylation of myrAkt1 was resistant to PLD inhibition, we next determined whether autophagic flux was restored following myrAkt1 expression in PLD inhibitor treated cells. Expression of myrAkt1 produced a modest increase in the basal level of LC3-II versus the parental U87MG line (Figure 16C). However, the fold induction of LC3-II due to PLD inhibitor treatment versus vehicle control was significantly less than in the parental U87MG line (Figure 16C and D), suggesting that the decrease in

autophagic flux was due to inactivation of Akt via a PLD dependent mechanism. Mechanistically, expression of myrAkt1 should prevent the increased binding of rubicon to beclin1 following treatment with PLD inhibitors. Treatment of myrAkt1 U87MG cells with Akt inhibitor MK2206, but not VU0364739, increased beclin1 binding to rubicon even in the presence of myrAkt1 (Figure 16E and F), supporting the proposed mechanism that PLD2 inhibition results in the inactivation of Akt, which promotes the Rubicon-Beclin1 interaction and inhibits autophagic flux.

Finally, to confirm that the decrease in viability following PLD inhibition was due to inhibition of Akt, we measured U87MG cell viability in the parental and myrAkt1 lines. Restoration of Akt function significantly increased viability and protected the GBM cells from PLD inhibitor induced cell death (Figure 16G). Taken together, these data suggest that PLD activity is required for full Akt activation in GBM cells and that when inhibited, cells undergo autophagic death.

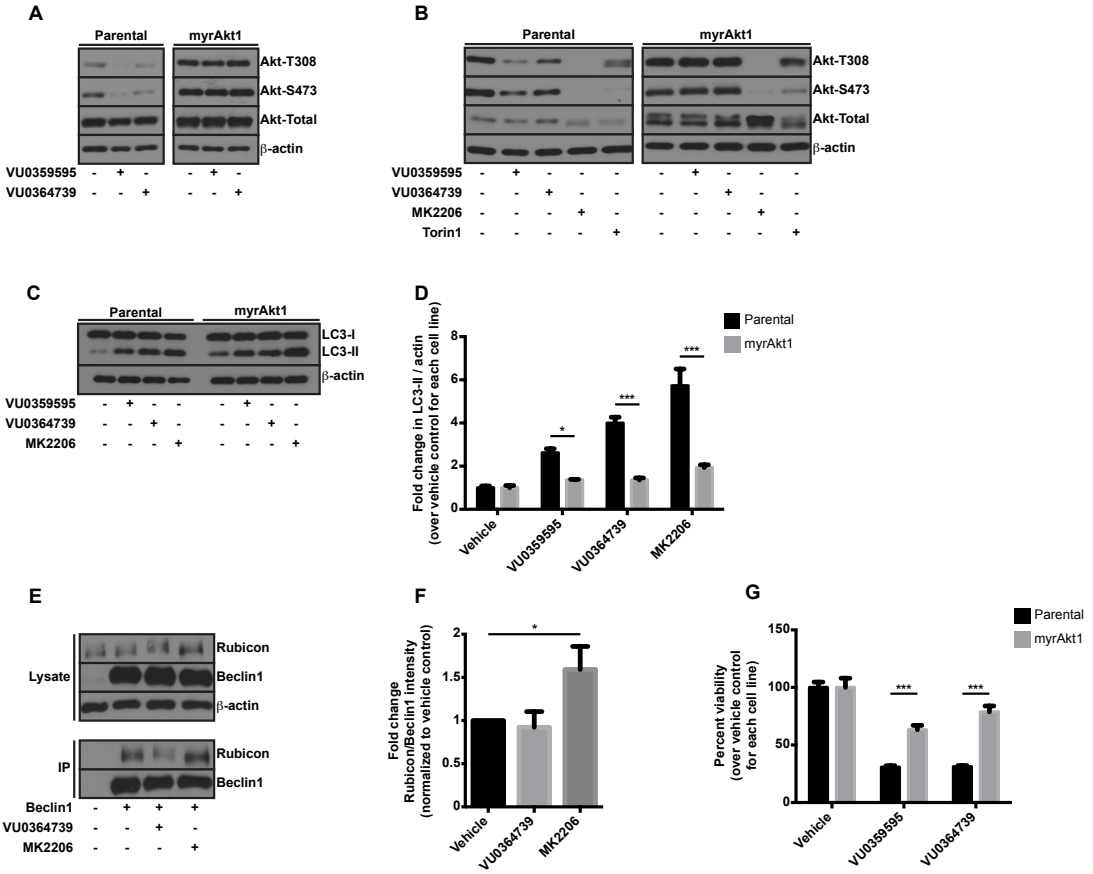

Figure 16. Restoration of Akt function rescues cell viability following PLD inhibitor treatment. **A**. Immunoblot of phosphorylated Akt from parental or myrAkt1-expressing U87MG cells treated overnight with 10 μM VU0359595 or 5 μM VU0364739 in serum-free DMEM. **B**. Parental or myrAkt1 U87MG cells were treated with the indicated inhibitors as in (A) overnight before blotting for phosphorylated and total Akt. **C**. Immunoblot of LC3 from parental or myrAkt1 U87MG following 6 hour treatment with PLD or Akt inhibitors in serum-free DMEM. **D**. Quantification of LC3-II conversion in parental and myrAkt1 U87MG cells following PLD and Akt inhibition. Fold changes were determined by calculating the ratio of LC3-II in inhibitor treated samples to vehicle treated samples within each cell line. * $p < 0.05$, *** $p < 0.005$, two-way ANOVA with Tukey's post-hoc test. **E**. MyrAkt1-U87MG cells were seeded and treated as in Figure 15B. **F**. Quantification of Rubicon binding to Beclin1 in myrAkt1 U87MG cells. Binding was quantified as in Figure 15C and * $p < 0.05$ using a paired student's t-test (n=4). **G**. WST-1 viability assay with parental or myrAkt1 U87MG cells treated for 24 hours with 20 μM VU0359595 or 10 μM VU0364739 in serum-free DMEM. Data is presented as the viability remaining following inhibitor treatment compared to the vehicle control within each cell type. *** $p < 0.005$, two-way ANOVA with Sidek's post-hoc test. Error bars - SEM.

Discussion

Although cancer research has improved the lives and long-term survival of many patients over recent decades, the prognoses and treatment options for patients with GBM remain grim. Treatment of drug-resistant GBM demands new understanding of the survival mechanisms used to sustain GBM growth and viability under stress conditions. The insight that most GBM are resistant to apoptotic stimuli has prompted investigators to examine autophagy as a survival mechanism that may be exploited for drug treatment (Lefranc et al., 2007). The chemotherapeutic drugs most successful in treating GBM, such as temozolomide, induce autophagy (Kanzawa et al., 2004). Drugs that interfere with autophagic flux, such as the anti-malarial drug chloroquine, have shown promise in clinical trials for potentiating cell death induced by conventional GBM chemotherapies (Sotelo et al., 2006). Therefore, identification of novel autophagy drug targets is clinically relevant for GBM.

In this chapter, we identified PLD, specifically the PLD2 isoform as a regulator of autophagy and cell survival in gliomas through its regulation of Akt kinase (Figure 17). Phosphorylation of Akt, under serum depleted conditions, requires PLD2-generated PtdOH for recruitment to membranes (Figures 3 and 11). The phosphorylation status of a constitutively membrane-associated Akt, myrAkt, is unperturbed by PLD inhibition (Figure 16*A* and *B*), suggesting that PLD2 functions to regulate Akt activation by membrane recruitment as opposed to modulating the activities of upstream kinases such as the mTORC2 complex, as has been suggested in other cell lines (Chen et al., 2012; Toschi et al., 2009). Additionally, we report that PLD2 and Akt co-IPfrom U87MG cell lysates and form a direct protein-protein interaction (Figure 4). Several Akt-interacting proteins have been identified and mediate a variety of effects including modulation of kinase activity and enhancing the ability of upstream kinases to activate Akt by rendering Akt a better kinase substrate (Du and Tsichlis, 2005). An interesting hypothesis emerges

in which PLD2-generated PtdOH recruits Akt to membranes and allows PLD2 to directly interact with Akt and regulate kinase activity or activation by upstream kinases, and this is the subject of ongoing investigation.

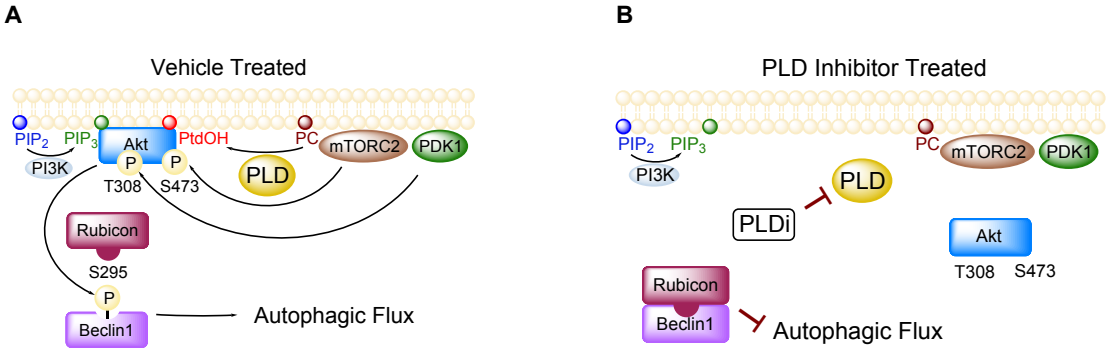

Figure 17. Mechanism of Akt and autophagy regulation by PLD. **A**. In the absence of inhibitors, PLD generates PtdOH and recruits Akt to the membrane allowing for phosphorylation of beclin1 by Akt at serine 295 and disruption of the Beclin1/rubicon complex and promotion of autophagic flux. **B**. PLD inhibitors reduce PtdOH production and subsequent Akt membrane recruitment. The inactivation of Akt results in reduced phosphorylation of beclin1 at serine 295 and formation of the beclin1/rubicon complex.

The coupling of PtdOH to Akt activation is apparent in GBM lines but not other cells such as the non-tumorigenic HEK293 line (Figure 3). The GBM cell lines used in this study, U87MG and U118MG, are both PTEN-null and have higher basal levels of Akt relative to other cell lines. Studies are emerging that suggest Akt-lipid binding profiles may be altered by post-translational modifications and could account for the differences we observe between various cell lines. For example, Mahajan et al. reported that phosphorylation at tyrosine 176 increases the affinity of Akt for PtdOH (Mahajan et al., 2010). In our study, PtdOH appears to increase the affinity of Akt for PIP_3 by binding a distinct site in the Akt PH domain, suggesting that PLD and PI3K might either work together or independently to activate Akt (Figure 11). Crystal structures of the PX domain from the NADPH oxidase protein $p47^{Phox}$ revealed a PtdOH binding pocket distinct from the well-characterized phosphoinositide binding site (Karathanassis et al.,

2002). Similar to our findings with Akt, occupation of the PtdOH binding pocket on $p47_{Phox}$ dramatically increased binding affinity for phosphoinositide-containing membranes (Karathanassis et al., 2002). These findings open the possibility that other lipid-binding proteins are subject to dual-regulation by PtdOH and phosphoinositides and expose the potential for alternative therapy strategies targeting one or both pathways as PLD and PI3K may function together to fine-tune subcellular localization and regulate specific effector pathways. In tandem, selective inhibitors might reduce the effective concentrations and thereby minimize undesired side effects.

Akt kinase has emerged as a regulator of autophagy and chemical inhibitors or genetic silencing can modulate autophagy in GBM cells (Degtyarev et al., 2008), although the mechanisms have not been fully elucidated. Here, we have characterized a novel function for the Akt-mediated phosphorylation of beclin1. Phosphorylation of Beclin1 by Akt appears to prevent binding to Rubicon (Figure 15*B* and *C*), an interaction that is known to inhibit autophagosome maturation (Matsunaga et al., 2009; Zhong et al., 2009). Further elucidation of novel Akt substrates in autophagy pathways will potentially shed light on other molecular mechanisms underlying the regulation of autophagy by PLD2 and Akt.

Our results support PLD as a novel drug target for GBM therapy. By regulating specific functions of Akt, such as autophagy (Figure 17), the side effects of global Akt inhibition (Carlson, 2013; Yap et al., 2011) are potentially avoided by targeting PLD2. Knockout mice for PLD1 and PLD2 (Elvers et al., 2010; Oliveira et al., 2010) are viable and show no overt phenotypes, suggesting that small-molecule inhibition of PLD may circumvent toxic side effects seen with conventional chemotherapies. Although we characterized the molecular mechanisms of cell survival in a model system, U87MG cells, PLD inhibitors decreased autophagic flux, reduced viability, and reduced anchorage independent growth in two glioma stem cell lines isolated from human

biopsies (Figure 2C and Figure 13F), which are notoriously resistant to conventional chemotherapies (Eramo et al., 2006). Therefore, the development of PLD inhibitors as a stand-alone or combination therapy has exciting potential for GBM treatment.

Materials and methods

Cell culture

myrAkt1-U87MG cells were maintained in DMEM + 10% tetracycline-free FBS (Atlanta Biologicals) + 1% P/S. Sf21 insect cells were obtained from Orbigen and maintained in Grace's Media (Life Technologies) supplemented with lactalbumin hydrolysate, yeastolate, sodium bicarbonate, and 10% FBS. Sf21 cells were maintained at 27 °C. Cell culture methods for other cell lines were described in chapter II.

Plasmids and baculovirus production

The following plasmids were obtained from Addgene: pcDNA3 T7 Akt1 (PI: William Sellers (Ramaswamy et al., 1999, plasmid 9003), pcDNA3 myr HA Akt1 (PI: William Sellers (Ramaswamy et al., 1999, plasmid 1036), ptfLC3 (PI: Tamotsu Yoshimori (Kimura et al., 2007, plasmid 21074), and pcDNA4 Beclin1-HA (PI: Qing Zhong (Sun et al., 2008b, plasmid 24399). FLAG-PLD1 and PLD2 were created by PCR amplification of the PLD open reading frames (PLD1 cDNA was obtained from Open Biosystems MGC collection, clone #6068382 and PLD2 cDNA was a generous gift from Dr. David Lambeth at Emory University) using forward primer containing FLAG epitope sequences and ligating into pcDNA5/TO (Life Technologies). To create the PtS-tagged PLD2 construct (PtS-PLD2), the PtS tag from p31-N-PtS (a kind gift from Dr. Yisong Wang (Giannone et al., 2007) was shuttled into pcDNA5/TO to create PtS-pcDNA5/TO and the PLD2 ORF was subsequently ligated 3' of the PtS ORF into PtS-pcDNA5 to create a

PLD2 construct with an N-terminal PtS tag. To create the PtS-PLD2 baculovirus, the PtS-PLD2 ORF was ligated into pENTR1A (Life Technologies). After LR recombination into pDEST8 (Life Technologies), baculovirus was produced according to manufacturer instructions. A bacterial expression vector for the PtS tag was created by amplification of the PtS tag from PtS-pcDNA5/TO and ligated into pET16b (EMD Millipore). To make PLD2.d308, amino acids 309-933 of human PLD2 were amplified and ligated into a pDEST derivative containing N-terminal His, MBP, FLAG, and TEV (HMFT) tags (Henage et al., 2006). HMFT-PLD2.d308 in pDEST8 was used to create bacmid DNA according to manufacturer instructions. PLD2-PXPH was created by amplifying the first 308 amino acids of PLD2 and ligating into pSV278 (Vanderbilt structural biology core). The pSV278 plasmid encodes a 6xHis-MBP tag 5' of a multiple cloning site so resulting constructs have an N-terminal tag.

For 6xHis-Akt1 and 6xHis-Akt1.d145 baculovirus production, the Akt1 ORF was amplified from pcDNA3 myr HA Akt1 and ligated into pENTR3C (Life Technologies). The full-length Akt protein encoded amino acids 2-480 and Akt.d145 encoded 146-480. pENTR3C was LR recombined into pDEST10 (Life Technologies) to generate a 6xHis-Akt1 construct and baculovirus was produced according to manufacturer instructions.

Transfection and RNAi

For protein expression, cells were transfected using Fugene 6 (Roche) according to the manufacturer instructions. All siRNA was obtained from Dharmacon as a pool of 4 oligonucleotide targeting sequences per relevant target (ON-TARGETplus). Cells were transfected according to manufacturer instructions using the Dharmafect 1 reagent and a final concentration of 100 nM siRNA.

Endogenous PLD activity assays

PLD activity assays were performed essentially as previously described (Brown et al., 2007). Following experimental treatments, cells were treated with 0.3% deuterated *n*-butanol (*n*-butanol-d_{10}) for 30 minutes prior to phospholipid extraction and quantification of PLD-generated phosphatidylbutanol species. To generate concentration response curves, U87MG cells were serum starved for approximately 24 hours. Cells were treated with the indicated concentration of PLD inhibitor for 15 min prior to addition of *n*-butanol-d_{10} and phospholipid extraction.

Viability assays

Cells were seeded into clear-bottom, black-walled 96-well tissue culture plates and allowed to adhere overnight to achieve approximately 60% confluence the following day. Cells were serum-starved in the presence of indicated inhibitors overnight. Viability was measured by addition of the WST-1 reagent (Roche) and reading absorbance at 450 nm. Time of WST-1 incubation was cell line dependent and ranged from 30 minutes to 2 hours.

To measure viability following RNAi treatment, cells were seeded in 60 mm tissue culture plates at 540,000 cells/plate and transfected with 100 nM siRNA. The next day, cells were split into 96-well plates, allowed to adhere overnight, and serum starved in the presence of inhibitors the following day. Viability was assessed approximately 72 hours post siRNA transfection.

Anchorage-independent growth assays

Base layers of 0.5 ml of 0.7% agarose (SeaKem GTG agarose, Cambrex Bio Science, Rockland, ME) containing complete neurobasal growth media were prepared in 12-well tissue culture plates. PLD inhibitors or DMSO vehicle controls were incorporated

into the base layers. A 0.5 ml overlayer of 0.35% agarose containing CD133+ cells (5.0 x 10^3) in compete growth medium with PLD inhibitors was applied. Each condition was plated in triplicate wells. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were fed every 2-3 days with complete growth media plus PLD inhibitor. Colony formation progressed for 8 weeks. Crystal Violet (0.005%) was used to stain colonies. Large colonies (>50 cells) were scored at 10× magnification with an inverted phase microscope using the average of 4 random fields per sample.

Immunoblotting

Lysates were prepared by incubating cell pellets in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 5 mM NaF, 1 mM DTT, and Roche complete protease inhibitor cocktail for 30 minutes at 4 °C. Protein concentrations were measured using the Bio-Rad protein assay reagent. Pan-Akt, Akt-S473, Akt-T308, beclin1, p62, Atg7, p70S6K, p70S6K-T389 antibodies were from Cell Signaling; GAPDH and PLD1 antibodies were from Santa Cruz; b-actin and FLAG (M2) antibodies were from Sigma; pan-cadherin and rubicon antibodies were from Abcam; and PLD2, HA-tag, strep-tag and LC3 antibodies were from Abgent, Covance, Qiagen and Novus, respectively. Bands were quantified using the gel analyzer function of ImageJ (NIH).

Protein purification

PtS-PLD2 infected *Sf*21 cells were harvested and collected by centrifugation at 500 × g for 5 min. Cells were lysed by sonication in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 0.5% Nonidet P-40, 2.5 mM EDTA, and 50 μg/ml Avidin, 1 mM DTT, complete protease inhibitor tablet (Roche), and 1 mM PMSF added immediately prior to sonication). Lysate was cleared by centrifugation at 12,000 × *g* for 10 min. Cleared

lysate was incubated with strep-tactin affinity resin (IBA) overnight at 4 °C. Beads were washed 3 times with wash buffer (lysis buffer with 0.01% Nonidet P-40). PtS-PLD2 was batch eluted by incubation of beads with 5 mM desthiobiotin (Sigma) in wash buffer for 10 minutes, centrifugation at 1,000 × *g*, and collecting supernatants containing soluble PtS-PLD2. For protein-protein interaction studies, PtS-PLD2 eluates were dialyzed (5,000 MWCO, Gibco) overnight against wash buffer to remove desthiobiotin.

The PtS protein, used as a control for protein interaction experiments, was produced by transforming BL21 *E. coli* (Agilent) with the pET16b-PtS plasmid. Bacteria were grown at 37 °C until OD_{600} reached 0.7. At that point, protein expression was induced by adding 100 μM IPTG and growing bacteria overnight at 18 °C. Bacteria were lysed by incubating in lysis buffer (30 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, complete protease inhibitor cocktail, and 1 mg/ml lysozyme) for 30 min followed by sonication. Lysates were clarified by centrifugation at 14,500 × *g* for 30 minutes at 4 °C and then loaded onto a 1 ml Hi-Trap chelating column (GE). The column was washed until OD_{280} returned to baseline and non-specific proteins were eluted using a 40 mM imidazole step gradient. Once OD_{280} returned to baseline, PtS was eluted in a linear imidazole gradient from 40-500 mM. Eluates were pooled and loaded onto a 120 ml Sephadex 75 gel filtration column (GE), previously equilibrated with 50 mM Tris pH 7.4, 0.5 mM EGTA, 150 mM NaCl, and 2 mM DTT. Fractions containing PtS protein were collected and pooled for use in binding assays.

For 6xHisMBP-PLD2-PXPH purification, BL21 *E. coli* were induced with 100 μM IPTG overnight at 18 °C. Cells were harvested by centrifugation at 6,000 × *g* for 15 minutes. Cells were lysed by incubation for 30 minutes in lysis buffer (30 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 1mg/ml lysozyme, Roche complete protease inhibitor cocktail, and 1 mM PMSF) followed by sonication. Lysates were clarified by centrifugation at 14,500 × *g* for 30 minutes. Cleared lysates were loaded onto a 5 ml

MBPtrap (GE) that had previously been equilibrated with lysis buffer minus protease inhibitors. The column was washed until OD_{600} returned to baseline at which time 6xHisMBP-PLD2-PXPH was eluted using a 10 mM maltose step gradient. Eluates were pooled and loaded onto a 1 ml HiTrap nickel chelating column that had been equilibrated in the maltose elution buffer. Column was washed until OD_{600} returned to baseline and non-specific proteins were eluted using a 40 mM imidazole step gradient. To elute PLD2-PXPH, an imidazole gradient from 40-500 mM was run over the column for 30 minutes. The fractions around the UV peak that eluted during the imidazole gradient were pooled (approximately 5 ml) and loaded onto a 120 ml sephadex 75 gel filtration column that had been equilibrated with 50 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, and 2 mM DTT. The column was run overnight at 0.25 ml/min. Correct protein folding was assessed by measuring binding of PLD2-PXPH to PI(3)P-containing protein-lipid overlays.

For PLD2.d308 purification, suspension cultures of *Sf21* insect cells were infected with HMFT-PLD2.d308 baculovirus for 72 hours before harvest. Cells were collected by centrifugation at 500 × *g* for 5 minutes. Cells were lysed by resuspending in lysis buffer (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.5, 137 mM NaCl, 2.5 mM KCl, 40 mM β-glycerophosphate, 0.5 mM DTT and Roche complete (EDTA free) protease inhibitor cocktail and sonicating. Lysates were clarified by ultracentrifugation at 100,000 × *g* for 45 min at 4 °C. Clarified lysates were loaded onto a 1 ml HiTrap nickel chelating column that had previously been equilibrated with lysis buffer minus β-glycerophosphate, DTT and protease inhibitors. The column was washed until OD_{600} returned to base line and non-specific proteins were eluted using a 32 mM imidazole step gradient. PLD2.d308 was eluted using a linear gradient from 32-400 mM imidazole. Elutates were pooled and concentrated to 0.5 ml and loaded onto a 24 ml Sephadex 200 (GE) gel-filtration column that had previously been equilibrated with 8.1 mM Na_2HPO_4, 1.5 mM

KH_2PO_4 pH 7.5,137 mM NaCl, and 2.5 mM KCl. Column was run at 0.5 ml/min to elute PLD2.d308.

N-terminal Akt PH domain 6xHis-GST fusion proteins were produced by PCR amplifying the first 123 amino acids of Akt1 (Thomas et al., 2002), ligating into pBG105 (Vanderbilt structural biology core), and transforming BL21 *E. coli*. Bacteria were grown at 37 °C until OD_{600} reached 0.7. At that point, protein expression was induced by adding 250 μM IPTG and growing bacteria overnight at 27 °C. Bacteria were lysed by incubating in lysis buffer (50 mM Tris buffer pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM DTT, Roche complete protease inhibitor cocktail, and 1 mg/ml lysozyme) for 30 min followed by sonication. Lysates were clarified by centrifugation at 14,500 × *g* for 30 min at 4 °C then applied to glutathione agarose (Sigma) for 2 h at 4 °C. Resin was washed twice with lysis buffer and twice with wash buffer (50 mM Tris pH 7.5, 300 mM NaCl, 0.1 mM EGTA, and 5 mM DTT) and eluted by incubating in wash buffer containing 10 mM reduced glutathione.

6xHis-Akt1 and 6xHis-Akt1.d145 was purified from Sf21 cells essentially as previously described (Kumar et al., 2001).

Immunoprecipitation and in vitro protein-protein interaction assays

Cells were resuspended in lysis buffer (see Immunoblotting section) and lysed with three freeze/thaw cycles in dry ice/ethanol, drawing lysate through a 25G syringe needle between cycles. Clarified (10,000 × *g* for 10 min) lysates were pre-cleared with protein-G agarose (Millipore) and immunoprecipitating antibodies were incubated with lysate overnight. Complexes were captured using protein-G agarose, washed three times in lysis buffer, then eluted by boiling in 2× SDS-PAGE loading buffer.

For *in vitro* protein-protein interaction assays, 25 nM PtS-PLD2, HMFT-PLD2.d308, 6xHisMBP-PLD2-PXPH, or protein tag controls were incubated with the indicated concentration of Akt construct for two hours in 50 mM Tris pH 8.0, 0.01% Nonidet P-40, 150 mM NaCl, and 0.5 mM EDTA. For strep-tactin pull-down experiments, 50 μg/ml avidin was included in the incubation buffer to reduce non-specific binding. Where indicated, 10 mM inhibitor was included in the reaction mixture. PtS-tagged proteins were captured by incubation with strep-tactin resin and MBP-tagged proteins were with amylose agarose for 2 h. Resin was washed 3 times and proteins eluted by boiling in 2× SDS-PAGE loading buffer.

In vitro Akt kinase assay

Kinase reaction mixtures included 50 nM 6xHis-Akt1, 100 mM ATP, and 125 ng GST-GSK3 substrate peptide (Cell Signaling) in a buffer containing 25 mM Tris pH 7.5, 5 mM β-glycerophosphate, and 2 mM DTT. Reactions were initiated by spiking a stock concentration of $MgCl_2$ to achieve a final concentration of 1 mM and reactions were incubated in a 30 °C water bath for 30 minutes. To terminate the kinase reactions, SDS-PAGE loading buffer was added to each tube and samples were boiled for 5 minutes. Kinase activity was determined by immunoblotting a portion of each sample with a phospho-GSK3 antibody (cell signaling). Where indicated, PLD2 proteins were included in the reaction mixtures at a final concentration of 50 nM.

Protein-lipid binding

The general procedure for measuring Akt binding to lipid spots on nitrocellulose membranes has been previously described (Dowler et al., 2002). The membrane containing various classes of lipids was obtained from Avanti Polar Lipids and contained 2 μg of the indicated lipid per spot. For vesicle competition assays, vesicles were prepared by drying lipids under N_2 gas and resuspending in 50 mM Tris pH 7.4, 150 mM

NaCl, and 2 mM EGTA. Lipids were vortexed and sonicated until in solution. The vesicles were composed of either 100% 32:0 PC or 95% PC + 5% 32:0 PtdOH (mol %). Recombinant Akt was incubated for 2 hours with 200 μM bulk vesicles, and then incubated with nitrocellulose membranes overnight. Lipid-bound Akt was determined using a total Akt antibody and chemiluminescence.

Exogenous PtdOH rescue

Lipids were obtained as chloroform solutions from Avanti Polar Lipids. Lipids were dried under N_2 gas in glass Pyrex tubes. Dried lipid film was vortexed in DMEM + 0.25 mg/ml fatty-acid-free BSA and then sonicated for 10 minutes. The sonicated lipid mixture was further diluted in the DMEM/BSA mixture to a final concentration of 1 mM and cells were treated for the duration of time indicated in the text.

Membrane isolation from U87MG cells

U87MG cells were seeded on 150 mm tissue culture plates at 2.6 × 10^6 cells/plate (4 plates per condition) and allowed to adhere overnight. The following day, cells were washed and media replaced with DMEM plus indicated inhibitor or vehicle and cells were treated for 6 hours. Cells were washed twice in 1x PBS then scraped in homogenization buffer (20 mM HEPES pH 7.4, 1 mM EDTA, 250 mM sucrose). Cells were pelleted by centrifugation at 1,000 × *g* for 5 minutes at 4 °C. Cell pellets were resuspended in homogenization buffer containing Roche complete protease inhibitor cocktail, 10 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4, and 5 mM NaF and lysed by nitrogen cavitation (1000 psi for 5 minutes, 4 °C). Lysate was collected dropwise then centrifuged at 2,000 × *g* for 10 minutes to pellet unbroken cells, nuclei, and heavy debris. The supernatant was subsequently centrifuged at 100,000 × *g* for 60 minutes. The supernatant was saved as the cytosolic fraction and the 100,000 × *g*

pellet was washed once by resuspension in lysis buffer then centrifuged again under the same conditions.

A stock iodixanol (Optiprep, Sigma) gradient solution was prepared by diluting the 60% iodixanol solution from the manufacturer in dilution buffer (120 mM HEPES pH 7.4, 250 mM sucrose, 6 mM EDTA, 60 mM β-glycerophosphate, 6 mM Na_3VO_4, and 60 mM sodium pyrophosphate) in a ratio of 5 parts 60% iodixanol to 1 part dilution buffer to create a 50% iodixanol working solution. 2.5%, 10%, 17.5%, 25%, and 30% iodixanol solutions were prepared by mixing the appropriate ratios of 50% iodixanol with homogenization buffer. Washed membranes from the second 100,000 × *g* spin were resuspended in 30% iodixanol (approximately 500 ml) and added to 11 ml polycarbonate ultracentrifuge tubes (Beckman Coulter). Equal volumes of 25%, 17.5%, 10%, 2.5% iodixanol were layered on top of the membrane suspension and tubes were centrifuged for 3.5 hours at 165,000 × *g* in a swinging bucket rotor. 1 ml fractions were collected from the bottom by introducing a small hole with a 25G syringe needle and collecting droplets. Samples were then boiled in 6× SDS-PAGE loading buffer prior to immunoblotting. Membranes predominantly banded at the 10%/17.5% iodixanol interface.

Immunofluorescence

U87MG-tfLC3 cells were seeded on glass coverslips in 6-well plates in complete media and allowed to adhere overnight. The following day, cells were washed and treated with inhibitors in serum-free DMEM for 24 hours. Cells were fixed for 15 minutes in 2% paraformaldehyde followed by washing in PBS. Coverslips were removed and mounted onto glass slides in Vectashield mounting media containing DAPI (Vector Labs). GPF/RFP images were acquired using Nikon A1R laser scanning confocal microscope equipped with a Plan Apo VC 60x 1.4 N.A. and 40× oil immersion lens.

Statistical analysis

Statistical analyses used for each figure are listed in the figure legend. Graphs of PLD activity and cell viability are representative from at least three experiments. Quantified immunoblots represent pooled data from at least three independent experiments unless otherwise noted in the text.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

When we initiated this project, significant progress had been made towards understanding the activators and inhibitors of PLD proteins. Many of these proteins, such as those involved in vesicular trafficking and cytoskeletal remodeling provided insights into the physiological roles of PLD. On the other hand, many of the molecular mechanisms for the cellular functions of PLD were not well characterized. The purpose of this project was to identify and characterize novel PLD-protein interactions to further understand the molecular mechanisms by which PLD promotes processes needed for cancer cell survival. The initial question for this project was to determine the common and unique binding partners for PLD1 and PLD2 following stimulation of a prototypical GPCR (M1 muscarinic receptor) and RTK (EGF receptor) in order to define specific signaling complexes. Although we did not detect unique protein complexes as a result of EGFR stimulation, our analyses identified novel PLD2-protein interactions that revealed a role for PLD in regulating cellular bioenergetics and in regulating Akt kinase to promote autophagic flux.

The discovery that PLD from *N. gonorrhoeae* interacts with and activates human Akt upon infection of human cervical epithelial cells (Edwards and Apicella, 2006) prompted us to investigate the regulation of Akt by human PLD in cancer. Since Akt is an important cancer-survival kinase, we hypothesized that PLD may promote cancer cell survival by regulating Akt activation and prompted us to investigate three main questions: How does PLD2 control activation of Akt kinase? How do PLD2 and Akt promote GBM cell survival? What are the molecular targets for PLD2-stimulated Akt activity? PLD-derived PtdOH targets Akt for membrane recruitment and activation in

GBM cells, leading to an autophagy-dependent cell death following inhibition of PLD. Downstream of PLD activation, Akt phosphorylates beclin1 and prevents the anti-autophagic interaction between beclin1 and rubicon. We also demonstrated a direct protein-protein interaction between PLD2 and Akt and presented data showing that PLD2 stimulates Akt kinase activity *in vitro*, independent of PLD2 lipase activity (chapter III). These data provide some molecular insight into how PLD regulates Akt-mediated GBM cell survival, and paves the way for several follow-up studies.

One of the unanswered questions regarding the PLD2-Akt interaction concerns the binding site of PtdOH on Akt. Since there is no canonical PtdOH-binding domain, either by sequence or structure (see Chapter I), determination of the PtdOH binding site will probably require mutagenesis or structural analysis. As shown in Figure 11 (Chapter III), PtdOH binds the Akt-PH domain in a location distinct from PIP_3-binding domain. Multiple crystal structures have been published of the Akt-PH domain, laying the groundwork for crystallizng Akt-PH bound to PtdOH. Additionally, NMR studies have provided structural insights into short chain PtdOH binding to mTOR (Veverka et al., 2007) and may also provide insights into the binding of PtdOH to Akt. Determination of the Akt PtdOH binding site may uncover a conserved PtdOH binding motif useful for the identification of unknown PtdOH binding proteins.

Another unanswered question regarding the PLD2-Akt interaction concerns the identification of Akt substrates regulated by the PLD2-Akt interaction. As shown in Figure 8 (Chapter III), PLD2 protein activates Akt kinase activity *in vitro* towards a substrate peptide. We proposed a scenario where PLD2 recruits Akt to membranes via PtdOH and then directly influences kinase activity via the PLD2-Akt protein-protein interaction. Treatment of glioblastoma cells with PLD inhibitors did not decrease phosphorylation of Akt substrates such as glycogen synthase kinase 3 (GSK3), TSC2, Bcl-2-associated death promotor (BAD), proline-rich Akt substrate of 40 kDa (PRAS40), or FoxO1.

Therefore, future experiments should use phosphoproteomic analyses to compare global phosphorylation status of known Akt substrates following inhibition of PI3K or PLD. In theory, these analyses may uncover PLD-specific regulation of known Akt substrates under certain conditions. Additionally, cells may be separated into various subcellular fractions to track co-localization of PLD2 and Akt. Phosphoproteomic analyses may identify PLD-regulated, Akt substrates in these fractions. Known Akt substrates can be immunoblotted with phospho-specific antibodies and other identified proteins may be searched bioinformatically for Akt phosphorylation consensus sequences as a starting point for further validation. These experiments may uncover uncharacterized Akt substrates that function independently of the canonical PI3K/Akt pathway through PLD. Additionally, the regulation of Akt by PLD and PtdOH may explain previously characterized effects on proteins such as p53. As mentioned in Chapter I, increased PLD activity is associated with a decrease in p53 levels. MDM2 controls p53 levels by targeting p53 for destruction. Since Akt is known to activate MDM2 (Mayo 2001), the increase in p53 degradation observed with increasing PLD activity may be explained by PLD promoting Akt activity. Characterization of Akt substrates regulated by PLD and/or PI3K will certainly enhance understanding of Akt-mediated cancer cell survival.

The loss of glioma viability following PLD inhibition appears to be mediated by inactivation of Akt as expression of constitutively active myrAkt protects cells from PLD inhibitor-induced cell death. Modulation of autophagy at either the initiation or degradation steps appear to have varying outcomes on cell viability when GBM cells are treated with compounds that induce autophagy. For example, GBM cell death induced by temozolomide (Kanzawa et al., 2004) or imatinib (Shingu et al., 2009) is potentiated by inhibition of autophagosome degradation with bafilomycin A1. Paradoxically, chemical or genetic inhibition of autophagosome initiation protects GBM cells against temozolomide (Kanzawa et al., 2004) or imatinib (Shingu et al., 2009) induced cell

death. In our study and others (Dall'Armi et al., 2010), PLD inhibition decreased autophagic flux and inhibition of autophagy initiation via siRNA knockdown of Atg7 protected U87MG cells against PLD inhibitor induced cell death (Figure 13H and I, chapter III). Therefore, PLD inhibition may result in the accumulation of cytotoxic molecules that must be cleared by autophagy for survival. The accumulation of autophagosomes containing cytotoxic cargo cannot be degraded following PLD inhibition or bafilomycin A1 treatment, due to inhibition of autophagic flux, and cell viability is ultimately compromised. A major unanswered question concerns the identification of the cytotoxic contents of these autophagosomes.

Mitochondria are frequently damaged during normal cell proliferation and by treatment with compounds such as those used for chemotherapy. Damaged mitochondria are cleared by a specialized autophagy, known as mitophagy, to maintain cell viability (Liu et al., 2009; Kim et al., 2007). Although we presented no data that directly links PLD activity to mitophagy, several lines of evidence from chapter II strongly suggest that PLD may regulate cellular bioenergetics at the level of the mitochondria. First, proteomic analysis of PLD2 complexes from HEK293-TREx-EGFR cells revealed a large list of putative mitochondrial interacting proteins. We confirmed the interaction between PLD2 and ATP synthase through immunoprecipitation experiments (Figure 6, chapter II), which suggested that PLD2 is at least partially localized to the mitochondria. However, the formation of a PLD2-ATP synthase complex following cell lysis does not necessarily mean the two proteins interact *in vivo*. Detergent solubilization could trigger non-physiological interactions between proteins that would normally be segregated through differences in subcellular localizations. Future studies should use subcellular fractionation techniques to determine if PLD co-localizes with mitochondria under experimental conditions used in chapter II. The second line of evidence for PLD participating in mitochondrial function is that proliferative signals such as serum and

growth factors increase ATP levels in a PLD-dependent manner in PTEN-null GBM and breast cancer cells, but not HEK293 cells. This second line of evidence answers two of our main questions from this chapter: What circumstances does PLD regulate intracellular ATP and which types of cells require PLD activity to maintain ATP levels? Non-dialyzed serum stimulated an increase in ATP whereas dialyzed serum stimulated both an increase in ATP and lactate, suggesting that non-dialyzed and dialyzed serum stimulates ATP synthesis through mitochondrial oxidative phosphorylation and glycolysis, respectively. PLD inhibition only blocks the non-dialyzed serum ATP response, suggesting that PLD functions at the level of the mitochondria (Figure 17, chapter II). Finally, inhibition of mitochondrial ATP synthesis with oligomycin upregulates glycolysis to produce the requisite ATP following serum stimulation (Figure 15, chapter II). PLD inhibitors block the oligomycin-induced increase in glycolysis, suggesting that PLD may function to relay signals about mitochondrial health (Figure 15, chapter II) and partially answering the final question from chapter III: What is the molecular mechanism of bioenergetic regulation by PLD? This hypothesis is partially substantiated by our observations that PLD2 activity is modulated by ATP and ADP (Figure 16, chapter II). When mitochondrial integrity is normal, mitochondrial ADP and ATP levels would be high and suppress PLD activity. Following mitochondrial damage, or oligomycin treatment, mitochondrial ADP/ATP levels would presumably decrease, and release the inhibitory clamp on PLD activity. Therefore, damaged mitochondria may rely on PLD activity to trigger mitophagy for cell survival. Whether PLD functions as a sensor of mitochondrial

Preliminary experiments suggest that mitochondrial morphology changes following treatment with our small-molecule PLD inhibitors. In U118MG cells, mitochondria display a filamentous morphology in serum-starved, vehicle-treated conditions. By contrast, the mitochondria of U118MG cells treated with VU0359595 and VU0364739 display a punctate morphology, similar to LC3 puncta seen in U87MG cells

following treatment with the same PLD inhibitors (Figure 1). Although these data are not conclusive, the mitochondrial morphology changes following PLD inhibitor treatment are consistent with mitophagy. A potential criticism of our work is that we did not address the possibility that mitoPLD mediates some of our observations in mitochondrial function. Previous studies using FIPI, a PLD inhibitor that shares a chemical scaffold to VU0359595, determined that mitoPLD is not inhibited by this series of PLD inhibitors (Su et al., 2009). Therefore, the effects on mitochondrial function and morphology following VU0359595 and VU0364739 treatment are most likely mediated by the conventional PLD isoforms.

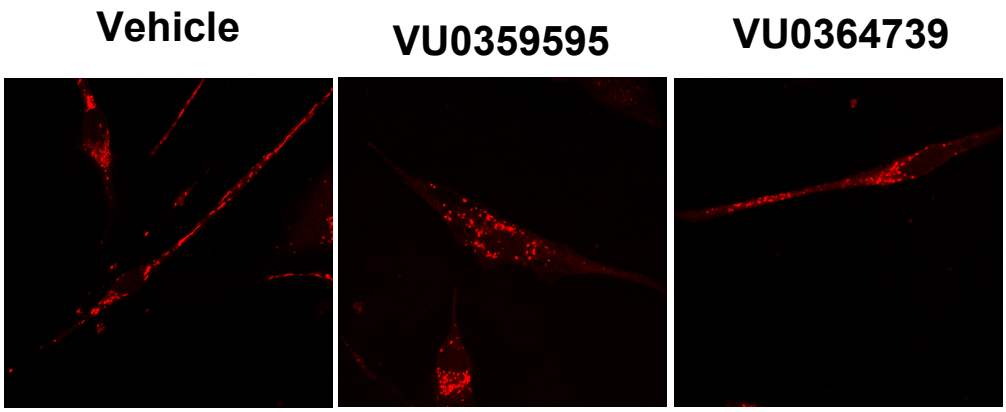

Figure 1. PLD inhibitor treatment results in altered mitochondrial morphology in U118MG cells. U118MG cells were serum-starved overnight and pretreated with 10 μM PLD inhibitor for 30 minutes prior to a 4-hour stimulation with 20% FBS. 100 nM MitoTracker Deep Red FM (Molecular Probes) was added to cell media during the final 30 minutes of the serum-stimulation. Cells were washed twice in PBS and fixed for 15 minutes in 2% PFA. Cells were mounted in Vectashield mounting media and imaged using an LSM 510 confocal microscope.

If the hypothesis that PLD functions as a bioenergetic sensor of mitochondrial health is true, future studies should focus on characterizing the downstream effectors of PLD in clearing damaged mitochondria. A recent study on mitophagy induction has suggested that leucine-rich pentatricopeptide repeat-containing protein (LRPPRC)

functions as a mitophagy suppressor (Zou et al., 2013). LRPPRC was first implicated in mitophagy following the observation that LRPPRC interacts with microtubule-associated protein 1S, a protein known to recruit LC3 to microtubules (Xie et al., 2011). When LRPPRC is silenced using siRNA, basal levels of mitophagy increase. Zou and colleagues demonstrated that LRPPRC interacts with beclin1 and prevents beclin1-induced activation of Vps34 to suppress autophagosome initiation (Zou et al., 2013). LRPPRC was identified in our proteomic screen as a PLD2 interaction partner (Table 1, chapter II). Although we have not validated the PLD2-LRPPRC interaction, an intriguing possible signaling pathway emerges. If PLD regulates LRPPRC localization or activity, then PLD activity could inhibit mitophagy through a positive regulation of LRPPRC. Likewise, PLD inhibitors would downregulate LRPPRC activity and promote mitophagy. This scenario is speculative, but may provide insight into the molecular mechanisms by which PLD inhibition induces mitophagy and subsequently prevents serum-stimulated ATP synthesis.

In addition to LRPPRC, PLD may signal through Akt to relay information concerning mitochondrial bioenergetic status. As discussed in chapter II, PLD inhibitors ablate the serum-induced increase in intracellular ATP. Similar results were observed with two structurally distinct Akt inhibitors, MK2206 and a dual Akt1/2 inhibitor, Akti1/2 (Lindsley et al., 2005). When U118MG cells were pretreated with either Akt inhibitor, the cells did not increase intracellular ATP when stimulated with ATP (Figure 2). Additionally, endogenous PLD2 and Akt were found to co-IP with endogenous ATP synthase α, suggesting that PLD2, Akt, and ATP synthase exist in a protein complex (Figure 2C). Phosphoproteomic characterization of Akt substrates in mitochondrial fractions may yield insight into the mechanisms by which PLD participates in cellular bioenergetics.

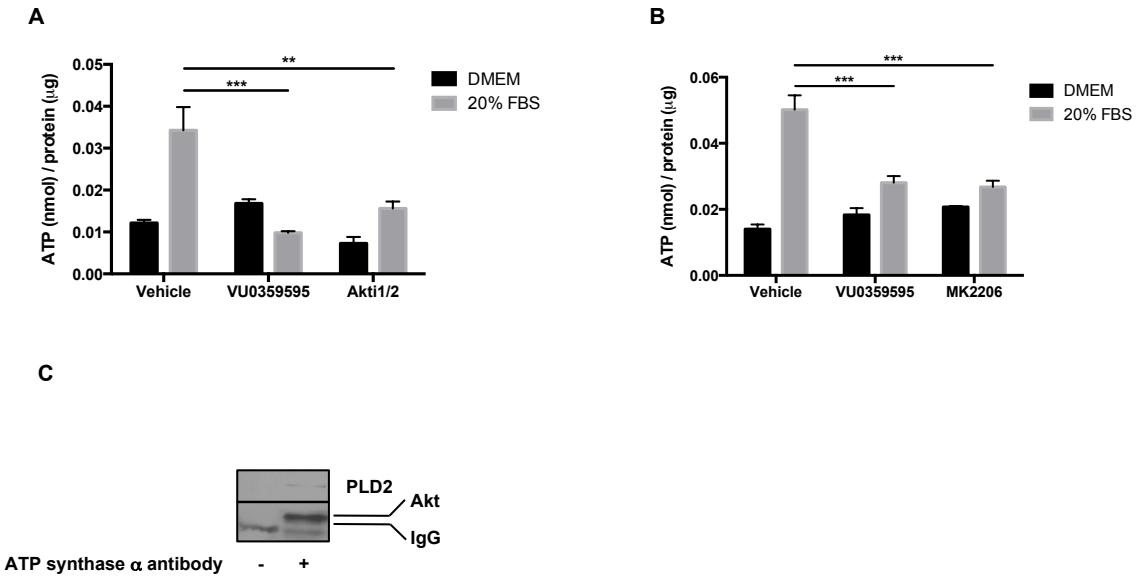

Figure 2. Akt participates in the serum-induced increase in intracellular ATP in U118MG cells. **A** and **B**. U118MG cells were serum deprived overnight and pretreated with 10 μM VU0359595, Akti1/2, (A) or MK2206 (B) for 30 minutes before a 4-hour stimulation with 20% FBS. Data is presented as the ratio of ATP to total cellular protein and representative experiments are shown with triplicate samples in each condition. ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey's multiple comparison test comparing FBS-stimulated ATP levels between vehicle and PLD/Akt inhibitor groups. **C**. PLD2 and Akt form a complex with ATP synthase. HEK293-TREx cells were serum-starved overnight before lysis the following day. Endogenous ATP synthase α was immunoprecipitated and complexes were probed for co-IP of endogenous PLD2 and Akt. An antibody of the same isotype as ATP synthase α served as a negative IP control.

The research contained herein has provided the tools to further understand the mechanisms by which PLD regulates cancer cell survival. This project may be used as a starting point for future studies to analyze PLD complexes in cancer cells versus non-cancer cells in order to gain insight into cancer-cell specific PLD complexes. Analyzing PLD complexes under stress conditions, such as serum-starvation or stimulation with apoptotic stimuli may also pave the way to identifying other PLD targets that promote cancer survival and chemotherapy resistance. Until then, by characterizing the regulation of Akt by PLD in GBM, we have provided a novel means of therapeutic intervention without directly targeting the Akt kinase.

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