REGULATION OF STRESS-INDUCED PHOSPHOLIPASE D SURVIVAL SIGNALS IN HUMAN CANCER

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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THE CITY UNIVERSITY OF NEW YORK

Abstract

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by

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Phospholipase D (PLD) is a phosphodiesterase that hydrolyzes phosphatidylcholine into phosphatidic acid (PA) and choline. In untransformed cells, PLD activity is stimulated by serum. In contrast, PLD activity in some cancer cells is stimulated by the stress of serum deprivation. The survival, migration and invasion of these cells depend on PLD activity. Thus, targeting the signals that elevate PLD activity may provide a tool for eradicating cancer cells that are able to survive insufficient serum conditions. Hence, we set out to determine which factors played a role in regulating stress-induced PLD activity. We focused on proteins such as EGFR and the G-proteins Ras, RalA, Arf1 and Arf6 which have been shown to regulate PLD activity. Correlating with PLD activity, we observed an increase in the activation of EGFR and Ras but not RalA in low serum. Pharmacological inhibition or siRNA knock down of EGFR abrogated the stimulated PLD activity. Comparatively, inhibition of Ras activity by dominant negative mutants or siRNA knockdown also suppressed PLD activity. Additionally, suppression of Arf1 and Arf6 by dominant negative mutants resulted in a decrease in PLD activity.

We next examined the effect of honokiol, a compound extracted from the Magnolia plant species, on PLD signaling. Honokiol suppressed PLD activity in cancer cells where PLD has been shown to suppress apoptosis. Importantly, the PLD activity induced by the stress of serum-withdrawal was selectively inhibited by honokiol. Honokiol was also shown to suppress Ras activation.

Another protein of interest to our work is 5`AMP- activated protein kinase (AMPK). This serine–threonine kinase senses and responds to changes in the AMP:ATP ratio. Interestingly, PLD activity was stimulated by the AMP mimetic and AMPK activator AICAR in high serum conditions. Pharmacological inhibition or siRNA knock down of AMPK prevented the induction of PLD activity. Suppression or activation of AMPK did not affect the activity of Ras. Similarly, inhibition of EGFR had no effect on AMPK activity. Pharmacological inhibition of AMPK caused an increase in cell death and an observed decrease in migration.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AMPK: 5’ activated protein kinase

AICAR: 5-aminoimidazole 4-carboxamide riboside

Cl PARP: cleaved PARP

DAG: diacylglycerol

EGF: epidermal growth factor

Hon: honokiol

PA: phosphatidic acid

PBt: phosphatidylbutanol

PC: phophatidylcholine

PDGF: platelet derived growth factor

PI3K: phosphoinositide 3-kinase

PIP_2: phosphatidylinositol-4,5-bisphosphate

PLD: phospholipase D

PMA: phorbol 12-myristate-13-acetate

PIP4-P-5K: type I phosphatidylinositol 4-phosphate 5-kinase

CHAPTER I

INTRODUCTION

1.1 PHOSPHOLIPASE D

1.1.1 PLD history

Phospholipase D (PLD) is a phosphodiesterase that catalyzes the hydrolysis of phosphatidylcholine into phosphatidic acid (PA) and choline. PLD-like activity was first described by Hanahan and Chaikoff in the late 1940s when they postulated that carrots and cabbage leaves contained an enzyme that was able to 'split' choline from phospholipids [1, 2]. However, evidence for the presence of PLD activity in mammals was not described until 1975 when Saito and Kanfer observed the hydrolysis of phosphatidylcholine into PA and choline in rat brain preparations [3]. The subsequent purification of PLD in the early 1990s from cabbage [4] and castor bean [5, 6] led to the cloning of PLD enzymes from various plants [7-9], yeast [10-13] and mammals [14-18].

1.1.2 PLD family and structure

Two isoforms of PLD (PLD1 and PLD2) have been identified in mammals and their amino acid sequence is approximately 51% identical [19, 20]. The 120 kDa PLD1 [15] which has low basal activity and 106 kDa PLD2 [20] whose basal activity is high, share homology in key motifs that have been shown to be essential for activity, (Fig. 1.1.2-1, [21]). To begin with, both PLD1 and PLD2 contain two HKD domains which are critical for enzymatic activity. These HKD domains contain the amino acid sequence HxxxxKxD, where the amino acids are Histidine (H), any amino acid (x) and aspartic acid (K). Point mutations in either one of the HKD domains in PLD1 or PLD2 renders the protein inactive [22]. This reliance on the charged amino acids within the PLD active site has led to a hypothetical reaction model which involves a covalent phosphatidyl-

enzyme intermediate (Fig. 1.1.2-2, [22]). In this model, a proton is donated from serine at position 911 (S911) to the C-terminal histidine and a proton is freed from the N-terminal histidine to create choline and a PA-PLD intermediate. Next, a proton is transferred from a water molecule to the N-terminal histidine, allowing the resulting hydroxyl group to hydrolyze the intermediate to form PA. Finally, S911 recovers its proton from the C-terminal histidine. This model has yet to be verified by structural data. Another motif, called the plekstrin homology (PH) domain, plays a role in the localization of PLD since deletion or point mutations in this domain results in mislocalization of the protein [23, 24] but does not alter its enzymatic activity [25, 26]. This observation led to the detection of the phosphatidylinositol-4,5-bisphosphate (PIP_2) binding motif on the PLD isoforms [23, 27, 28]. An additional motif, called the phox (PX) consensus sequence, is believed to mediate protein-protein interactions or to bind phosphatidylinositol phosphates (PIP) [28, 29]. Finally, PLD1 contains a loop region that is absent in PLD2. This loop region has been proposed to function as a negative regulatory element since its deletion results in a 3-fold increase in basal PLD1 activity [25].

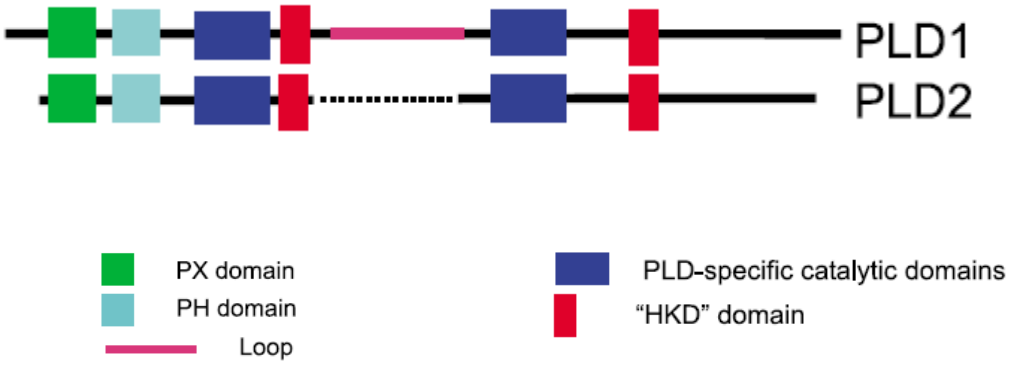

Figure 1.1.2-1: Basic structure of mammalian PLD1 and PLD2. Linearized cartoon showing conserved domains as boxes. Domains shown are the phox consensus sequence (PX), plekstrin homology (PH), catalytic HKD, PLD-specific catalytic domains and the PLD1 loop region. (Modified from [21])

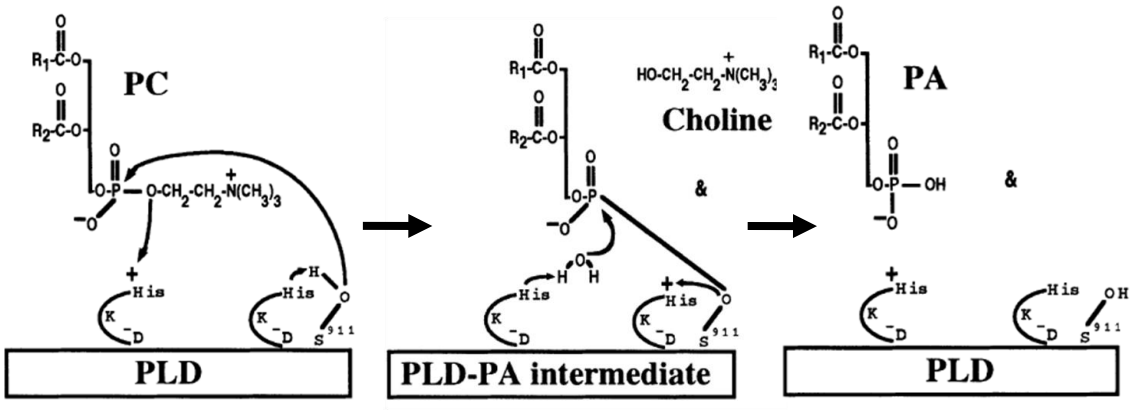

Figure 1.1.2-2: A PLD catalytic model. At the beginning of the reaction cycle, the N-terminal HKD is protonated. In the first half-reaction a covalent linkage is formed between PA and S911 which produces a PLD-PA intermediate with the concomitant liberation of choline. In the final half-reaction water hydrolyzes the intermediate to form PA [22].

1.1.3 PLD localization

Reports on the subcellular localization of PLD1 and PLD2 contain varying results. Overexpression studies performed by Colley et al. showed that in rat embryonic fibroblasts, PLD1 localized primarily to perinuclear regions of the cell with a pattern consistent with that of the Golgi apparatus, endoplasmic reticulum (ER) and endosomes [20]. However, studies in rat pituitary tumor cells by Freyberg et al. showed that endogenous PLD1 had a diffuse distribution which included nuclear staining and PLD1 also colocalized with markers of the Golgi [30]. In contrast, overexpression studies by Toda et al. and Hughes et al. found that PLD1 did not localize to the Golgi or the ER but instead was found in late endosomes and lysosomes only [31, 32]. Others have found PLD1 at the plasma membrane in cells stimulated by either phorbol 12-myristate-13-acetate (PMA) [28], insulin [33] or other agents [34]. On the other hand, most reports demonstrate that PLD2 localizes to the plasma membrane [20, 35], but it has been found in the cytosol [36], submembranous vesicular compartments [37] and associated with β-actin [38]. PLD2 has also been shown to translocate to and from the plasma membrane in cells stimulated by serum [20] and the epidermal growth factor (EGF) [36].

1.1.4 PLD regulation

PLD activity is regulated by a plethora of factors which include Ca^{2+}, protein kinase C (PKC), tyrosine kinases, phosphoinositides, fatty acids, growth factors and their respective receptors and small GTP binding proteins [39] (Table 1.1.4).

Calcium

Evidence to demonstrate a link between Ca^{2+} and PLD activity was provided by several reports in which PLD activity was stimulated by the ionophores A23187 and ionomycin in a variety of cells and tissues [40-44]. Comparatively, other studies have shown that depletion of cellular Ca^{2+} by the chelators ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA/AM) caused a decrease in the activation of PLD by various agonists [39, 45-47]. Many hypothesize that these observations may be mediated by Ca^{2+} sensitive proteins that regulate PLD [48].

PKC

Observations that PLD activity can be stimulated by phorbol esters such as the stable diacylglycerol (DAG) analogue PMA, indicated that PLD may be regulated by Ca^{2+}-sensitive PKC [21, 49]. Subsequent studies using specific [50] or non-specific [51, 52] inhibitors of PKC proteins showed either a partial or complete inhibition of PLD by agonists in several cell types. Comparatively, overexpression of several PKC isoforms resulted in an enhancement of agonist stimulated PLD activity in fibroblasts [53-57]. In contrast, our lab has demonstrated that inhibition of PKCδ enhanced the PLD activity of Rat 3Y1 cells overexpressing the EGF receptor (EGFR) [58].

PIP_2

Another molecule implicated in the regulation of PLD is phosphatidylinositol(4,5)-bisphosphate (PIP_2). PIP_2 strongly stimulated PLD activity in

HL60 cells [59] and depletion of PIP_2 with neomycin inhibited membrane bound PLD activity. This loss of PLD activity was restored by the re-addition of PIP_2 [60]. Other reports show that the catalytic activity of PLD1 and PLD2 are strongly dependent on PIP_2 [15, 18-20, 61]. PIP_2 is produced primarily from phosphatidylinositol(4)-phosphate (PI(4)P) by the type I phosphatidylinositol 4-phosphate 5-kinases (PIP4-P-5K) [62]. Work done in Cos7 cells demonstrated that PLD activity can be stimulated by the overexpression of PIP4-P-5K 1α and that both PLD1 and PLD2 physically associate with this kinase [37].

Fatty acids

Unsaturated fatty acids have also been implicated in the regulation of PLD activity. For instance, Kim et al. showed that PLD2 activity can be stimulated by the unsaturated fatty acids oleate, linoleate and arachidonate but not by the saturated fatty acids myristate, palmitate, stearate or arachidate [63].

Growth factors and tyrosine kinases

Mammalian PLD proteins are highly responsive to growth factor and hormone stimulation [64]. Numerous reports provide evidence which show that PLD activity can be stimulated by epidermal growth factor (EGF) [65-68], platelet derived growth factor (PDGF) [69, 70], fibroblast growth factor (FGF) [71, 72], vascular endothelial growth factor (VEGF) [73] and insulin [52, 74-77] in various systems. In some of these cases, PLD proteins have been shown to physically associate with the receptors of the aforementioned growth factors [78, 79]. Inhibitors of tyrosine kinases such as genistein inhibited PLD activity stimulated by various agonists [80-82]. On the other hand,

inhibition of protein tyrosine phosphatases with compounds such as vanadate, stimulated PLD activity [82].

G proteins

Work done by various labs in the early 1990s demonstrated that PLD activity could be stimulated by guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) a non hydrolysable analogue of GTP [83-86]. Subsequent studies revealed that this activation of PLD was mediated by guanine nucleotide binding proteins such as ADP-ribosylation factor (ARF) [59] and RhoA. Of the six mammalian ARFs identified, four (ARF1, 3, 5 and 6) were reported to activate PLD1 with equivalent efficacy [21]. Inhibition of certain ARF-GEFs by Brefeldin A or introduction of dominant negative mutants of ARF1 and ARF6 suppressed the activation of PLD by PDGF and PMA [87]. Other relevant G proteins that have been implicated in PLD activity such as Ras and RalA will be expounded in subsequent sections.

PLD inhibitors

Several proteins have been reported to negatively regulate PLD activity. They include but are not limited to synaptojanin, fodrin, and clathrin assembly protein-3 (AP-3). Synaptojanin is a member of the inositol polyphosphoinositide 5-phosphatase family which functions as a nerve terminal protein and has been implicated in the endocytosis of fused synaptic vesicles. This phosphatase produces this inhibitory effect by hydrolyzing the PLD activator PIP_2 [88]. Lukowski et al. reported that fodrin, a non-erythroid form of spectrin, inhibited PLD activity stimulated by various agents. This inhibitor works by sequestering PIP2 [89]. Finally, AP-3, a synapse specific protein, binds to and inhibits

PLD1. This data suggests that inhibition of PLD1 activity may play a role in the AP-3 dependent rapid cycling of synaptic vesicles [90].

Table 1.1.4: Characteristics of PLD isoforms

	PLD1 (120 kDa)	**PLD2 (106 kDa)**
Substrate specificity	PC	PC
PIP_2-dependent	yes	yes
RalA- associated	yes	no
Basal activity	low	high
Subcellular localization	Mainly intracellular membranes	Plasma membrane
Activators	ARF, PKC, Rho	Unsaturated fatty acids

1.1.5 PLD signaling

Although PLD catalyzed reactions produce choline and phosphatidic acid (PA), it is widely believed that PLD exerts its biological effects through PA. In addition, PA can be reversibly converted into DAG and lysophosphatidic acid (LPA) through the actions of phosphatidic acid phosphohydrolases (PAPs) [91, 92] and phospholipase A (PLA) [93] respectively. The reverse reactions that convert DAG and LPA into PA are carried out by diacylglycerol kinases (DGK) and lysophosphatidic acid acyltransferases (LPAAT) respectively (Fig. 1.1.5, [94]).

The unique ability of PLD enzymes to divert the formation of PA to the metabolically stable phosphatidyl alcohol has proven to be a vital tool in identifying PLD-dependent events. As a result, PLD has been shown to play a role in cellular

processes such as lipid biosynthesis [95], membrane trafficking (e.g. exocytosis/endocytosis) and signaling cascades that regulate cell growth, migration, proliferation, survival and differentiation [21, 96]. Proteins such as phosphatidylinositol 4-phosphate-5-kinase (PIP4-P-5K) [97, 98], Raf-1 protein kinase [99] and the mammalian target of rapamycin (mTOR) [100] are known to interact with and depend on PA for their activities.

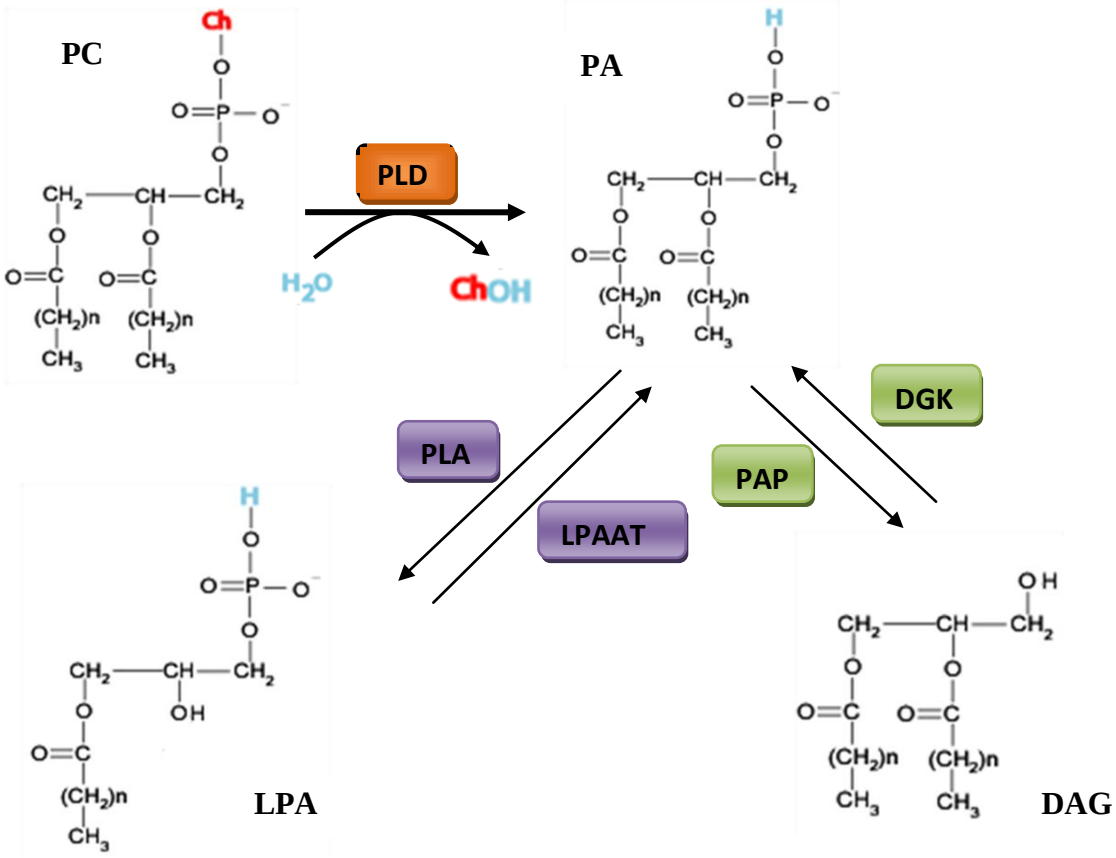

Figure 1.1.5: **Creation of second messengers from phosphatidyl choline (PC)**. PC is hydrolyzed to form choline (ChOH) and phosphatidic acid (PA), the latter of which is then reversible converted to diacylglycerol (DAG) and lyso-phosphatidic acid (LPA). Enzymes abbreviated are phospholipase D (PLD), phosphatidic acid phosphohydrolase (PAP), diacylglycerol kinase (DGK), phospholipase A (PLA) and lyso-phosphatidic acid acyltransferase (LPAAT). (Adapted from [94]).

1.1.6 PLD in cancer

Transformation

A role for PLD in transformation of mammalian cells has been established by several groups. The soluble protein kinase v-Src, transforms fibroblasts with a resulting increase in PLD activity [101]. By contrast, overexpression of c-Src, which did not elevate PLD activity [102], also did not transform cells unless it was combined with elevated expression of either PLD1 or PLD2 [103]. Likewise, elevated expression of PLD1 or PLD2 transformed rat fibroblasts overexpressing the EGF receptor [103, 104]. Additional data by Buchanan et al. [105] showed that transformation by H-RasV12 required PLD activity while Min et al. [106] demonstrated that cells overexpressing either PLD isozyme displayed anchorage independent growth and formed tumors in nude mice. PLD activity was also stimulated in cells transformed by oncogenes such as v-Src [101] and v-Fps [107]. The evidence provided here support a correlation between PLD activity and transformation.

Survival

Programmed cell death, otherwise known as apoptosis, has been associated with PLD function. The induction of apoptosis in various cell lines with agents such as actinomycin D and tumor necrosis factor –alpha (TNF-α), resulted in an increase in PLD activity [108, 109]. However, others reported a decrease in PLD activity when apoptosis was induced [110, 111], an effect that was reversed by overexpression of PLD1 and PLD2 [112]. Additional antiapoptotic effects of PLD1 and PLD2 have been described in transformed and breast cancer cells. Specifically, the serum-withdrawal induced

apoptosis of c-Src overexpressing 3Y1 rat fibroblasts was reversed by overexpression of either PLD isoform. In that same report, v-Src transformed 3Y1 cells were resistant to apoptosis induced by serum-withdrawal. The MDA-MB-231 breast cancer cell line exhibits elevated levels of PLD activity. Apoptosis in response to serum-withdrawal was obtained when PLD activity was inhibited [113]. Overall, the data indicate that PLD is required for survival.

PLD in metastasis

The ability of cancer cells to spread, commonly referred to as metastasis, plays a critical role in predicting cancer malignancy. In order to metastasize, cancer cells must develop two key abilities-increased motility and invasiveness. Cell motility or migration, is driven by molecules within the cytoskeleton including microtubules, actin and intermediate filaments [114]. Reports that indicate a link between PLD and migration show that PLD activity contributes to the reorganization of the actin cytoskeleton [115-117], the spreading of murine cells [118] and the formation of cell protrusions in v-Src transformed cells [119]. In addition, Rho GTPases, which have been shown to regulate migration [120], also activate PLD1 [121]. The secretion of proteases to degrade tissue barriers is a key component of cell invasion [122, 123]. PLD activity has been correlated with the secretion of proteases such as MMP-2 and MMP-9 in metastatic cells [124-127]. In addition, work done by Imamura et al. [128] and Pai et al. [129] further implicate PLD activity in tumor invasion. Most notably, like the PLD activity, the migration and invasion of the breast cancer cell line MDA-MB-231 was stimulated by serum withdrawal. Upon inhibition of PLD, there was a marked reduction in survival, migration and invasion [113, 130]. Taken together, this data suggests that PLD contributes to cell

motility and invasiveness, indicating an important role for PLD in the metastasis of cancer.

1.1.7 PLD as a therapeutic target

The involvement of PLD in so many hallmarks [131] of tumorigenesis indicate that targeting PLD or PLD signaling could prove valuable in the treatment of cancers exhibiting elevated PLD activity. In support of this theory, it has been reported that PLD activity is elevated in human breast cancer [132], experimental colon cancer [133], human gastric cancer tissue [134] and human renal cancer [135] when compared to adjacent normal tissue. In addition, elevated PLD activity confers resistance to the anti-proliferative drug rapamycin, while decreased PLD activity renders cells sensitive to rapamycin [136]. The data here reinforces the idea that PLD is a propitious target.

1.2 EPIDERMAL GROWTH FACTOR RECEPTOR

1.2.1 EGFR history

The epidermal growth factor (EGF) receptor (EGFR) is a member of the erbB family of receptor tyrosine kinases and plays a critical role in the transduction of signals from the cell surface to key molecules that regulate cell proliferation, survival, adhesion, migration and differentiation [137]. The first effects of EGF were demonstrated by Stanley Cohen who showed that treatment of mice with EGF induced precocious eyelid opening and tooth eruption [138]. The subsequent isolation and study of the human epidermal growth factor (EGF) in 1975 [139, 140] led to the purification of EGFR [141]. Binding of cognate ligands to EGFR induces dimerization which leads to receptor autophosphorylation and activation of signal transduction pathways. Whereas growth factor induced EGFR signaling is critical for normal morphogenic processes, aberrant activity of members of the erbB family plays a key role in the development of tumor cells [137].

1.2.2 EGFR family and structure

The 170 kDa EGFR/erbB1 is a membrane bound glycoprotein that belongs to the erbB family of receptor tyrosine kinases that also includes HER2/erbB2, HER3/erbB3 and HER4/erbB4 [142]. EGFR is composed of an extracellular ligand binding domain, a single transmembrane region, an intracellular kinase domain and a C-terminal tail with multiple phosphorylation sites (Fig. 1.2.2, [143]). The amino terminal extracellular domain contains two cysteine-rich regions responsible for binding to ligands [144] while the remainder of the extracellular domain plays a role in receptor dimerization and

interaction with other membrane proteins [145]. The transmembrane domain is believed to serve as a site for feedback attenuation by PKC and extracellular signal-regulated kinase/mitogen activated kinase (ERK, MAPK) [146, 147]. Additional evidence suggests that a motif within this region may link to heterotrimeric G proteins [148, 149]. Next comes the tyrosine kinase domain followed by the five autophosphorylation motifs within the carboxy-terminal tail [146]. These autophosphorylation motifs link to proteins containing SH2 or phospho-tyrosine binding (PTB) domains [146, 150]. The C-terminal tail also contains three internalization motifs comprised of a right turn as well as sites for transphosphorylation, proteolytic activation and degredation [146]. This tail is also reported to function as an autoinhibitory substrate since either deletion or mutation of the autophosphorylation sites prevents the ligand-activated EGFR from phosphorylating downstream substrates [151, 152].

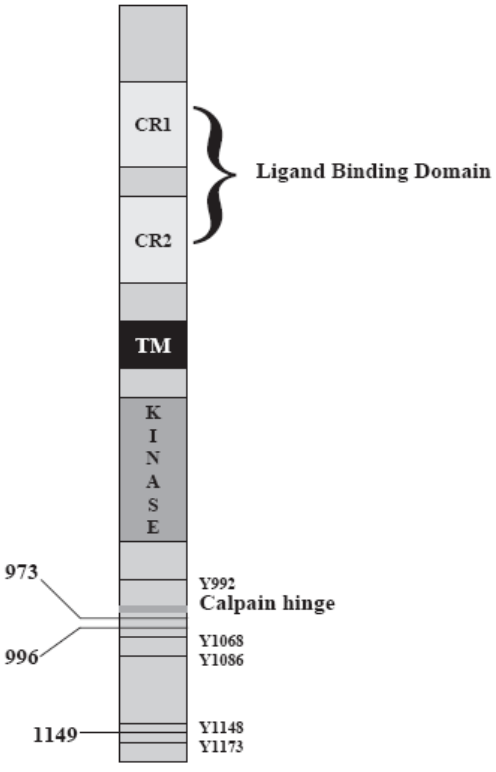

Figure 1.2.2: **Graphical representation of the mature EGFR.** The cysteine-rich CR1 and CR2 domains are involved in ligand binding of the receptor. The TM (transmembrane domain) separates the extracellular domain from the cytoplasmic tail. The cytoplasmic tail includes the tyrosine kinase domain as well as the autophosphorylated tyrosine (Y) residues and the calpain cleavage sitee. The amino acids 973, 996, 1149 are the three internalization domains [143].

1.2.3 EGFR regulation

Ligands

With the exception of HER2, whose ligand has not been identified, the erbB family receptors become activated upon binding to ligands. Several ligands bind to and activate EGFR including EGF, transforming growth factor α (TGF-α), amphiregulin and heparin-binding EGF (HB-EGF) [153]. EGFR ligands are synthesized as transmembrane precursors consisting of an EGF motif flanked by an N-terminal extension and a C-terminal membrane-anchoring domain [154]. This precursor is proteolytically cleaved, possibly by metalloproteases [155], to produce mature soluble ligands [154]. Specifically, the metalloproteases ADAM-17 and MMP-2, 3 and -9 have been shown to be involved in the processing of TGF-α and HB-EGF respectively [143].

Receptor stability

Upon binding of a ligand, the dimerized and activated EGF receptors are rapidly internalized and delivered to early endosomes, a process that is generally believed to downregulate growth factor signaling [156]. Once internalized in early endosomes, the ligand-EGFR complex is either targeted for ubiquitination and degradation or recycled back to the plasma membrane. Cbl is the primary E3 ubiquitin ligase that is recruited to the ligand-receptor complexes via a phosphorylated Tyr1045 residue [157]. The receptor is then targeted for lysosomal and proteasomal degradation since association with Cbl promotes receptor ubiquitination [137, 158]. In support of this hypothesis, reports show that in the absence of Cbl, receptors are recycled to the plasma membrane [158] and that mutation of Tyr1045 augments EGFR signaling [157]. The fate of the EGFR is also

determined by the stability of the activated ligand-receptor complexes in the acidic (pH5.5) endosomal environment (Fig.1.2.3, [137]). For instance, activated EGFR homodimers are relatively stable, remain bound to Cbl and are targeted for degradation. However, EGFR-HER2 heterodimers are rather unstable and uncouple in early endosomes. This results in the dissociation of Cbl from the receptor complex after which the receptors are returned to the cell surface through a default recycling pathway [159]. The type of ligand associated with the EGFR also determines the receptor's fate. For example, EGF remains bound to EGFR in the acidic late endosomes which directs the receptor for degradation. On the other hand, TGFα dissociates from EGFR in the acidic environment which results in recycling of the receptor [160].

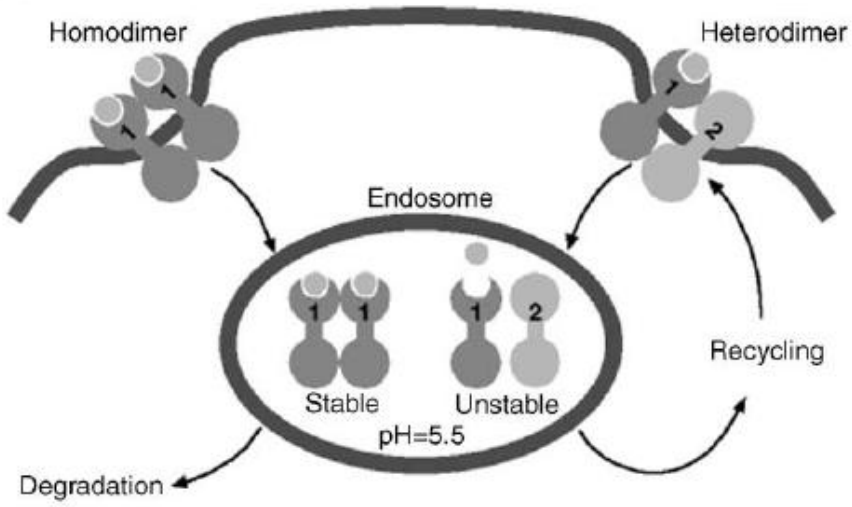

Figure 1.2.3: **Cartoon depicting EGFR fate.** The stability of the activated ligand-receptor complex in the endosomal environment affects receptor fate. Once in the sorting endosome, activated EGFR-HER2 heterodimers are relatively unstable. The ligand-receptor complex dissociates and the receptors are recycled back to the surface. In contrast, the stable EGFR homodimeric complexes are directed through an endocytic pathway that culminates in receptor degradation [137].

1.2.4 EGFR signaling

EGFR activation triggers a plethora of downstream pathways (Fig. 1.2.5), some of which include phosphoinositide 3-kinase (PI3K) and the small G protein Ras. Phosphorylation of tyrosine residues on the EGFR promotes binding of the SH2 adaptor protein Grb2 which in turn recruits SOS, a guanine nucleotide exchange factor (GEF) that activates Ras [161]. Active Ras in turn stimulates a variety of proteins such as MAPK, RalGDS and PLD [162]. These signaling cascades affect processes such as cell cycle progression, proliferation, survival, adhesion, migration and differentiation. Recently, it has been reported that EGFR protects cells from glucose-deprivation by increasing glucose uptake via the stabilization of the sodium/glucose cotransporter 1 (SGLT1) [163].

1.2.5 EGFR and PLD

A role for PLD in EGF/EGFR mediated signaling has been established by work done in our lab and others. To start, some of these reports showed that treatment of various cell lines with EGF stimulated PLD activity [58, 65, 66, 70, 80]. Interestingly, Slaaby et al. reported that PLD2 associated with the EGFR in a ligand-independent manner however, EGF-induced activation of the EGFR led to the phosphorylation of PLD2 [78]. Agonist-stimulated endocytosis and degradation of the EGFR receptor is dependent on PLD since overexpression of wild-type and mutant PLD proteins respectively accelerated or retarded receptor degradation [68]. In addition, dynamin, a large GTP binding protein that mediates receptor endocytosis, has been shown to interact with PLD2 in a GTP dependent manner [164]. This lab subsequently showed that the PX domain of PLD functioned as a GTPase activating protein (GAP) which was responsible

for the activation of the GTPase domain of dynamin [165]. A relationship between PLD activity and EGF induced migration was established by the report that the migration of epithelial cells induced by EGF was inhibited by 1-butanol, an effect that was reversed by the addition of phosphatidic acid analogues. Interestingly, this report also demonstrated that phosphatidic acid activated EGFR through a transactivation process [166]. Finally, our lab demonstrated that elevated PLD expression cooperated with overexpressed EGFR to transform 3Y1 rat cells [104].

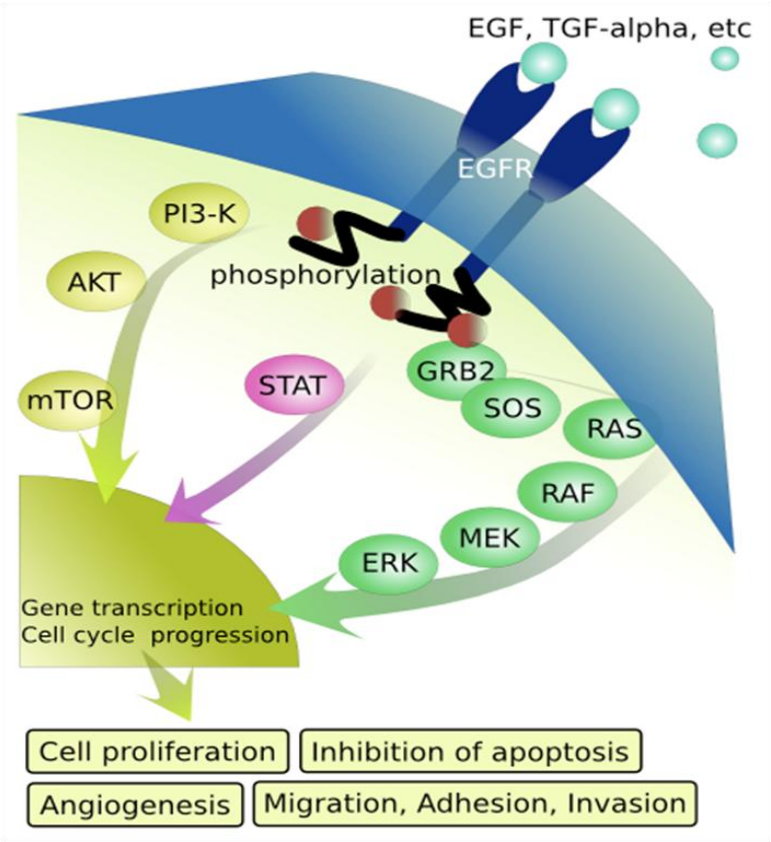

Figure 1.2.5: **Signaling network activated by ligand-dependent activation of EGFR.** Upon ligand-induced activation, EGFR selectively activates a plethora of signal transduction pathways that affect transcription, cell cycle progression, survival, proliferation, angiogenesis, adhesion, migration and invasion. [Source: http://commons.wikimedia.org/wiki/Image:EGFR_signaling_pathway.png]

1.2.6 EGFR as a therapeutic target in cancer

Aberrant EGFR signaling due to overexpression, mutation and autocrine growth factor loops, is a key feature of hyperproliferative diseases such as cancer [167]. Elevated EGFR expression was observed in many cancers including breast, head-and neck, non-small-cell lung (NSCLC), renal, ovarian, colon, and in a small percentage of gliomas, bladder and pancreatic cancers [168]. The expression of EGFR in normal cells ranges from 0.4×10^5 to 1×10^5 receptors per cell [169]. In striking contrast, some breast cancer cells express up to 1×10^6 EGFR receptors per cell [170, 171]. Elevated EGFR expression has been linked to advanced tumor stage, resistance to standard therapies such as chemotherapy and radiation [172-174], and poor patient prognosis [175], especially in NSCLC [176].

Consequently, EGFR was proposed as a rational target for antitumor strategies and several inhibitors have been developed that interfere with EGFR signaling. For instance, monoclonal antibodies exert their effects by interacting with the extracellular ligand-binding domain of EGFR while tyrosine kinase inhibitors (TKIs) function intracellularly by competitively binding to the receptor's ATP site [177]. Both of these drugs cause a disruption of the pathways that lead to tumor cell proliferation, angiogenesis, metastasis and cell survival [178, 179].

1.3 Ras

1.3.1 Ras history

Ras is a small guanosine triphosphate hydrolase (GTPase) that mediates a variety of cellular processes by translating extracellular signals into specific biological responses such as proliferation, differentiation and survival. From the late 1970s into the early 1980s, scientists studying the transforming activities of the rat-derived Harvey and Kirsten murine sarcoma retroviruses, discovered that the *ras* (rat sarcoma virus) genes contributed to the cancer pathogenesis of these retroviruses [180]. Subsequently, the cellular homologues of the retroviral transforming *ras* sequence were identified in the rat [181], mouse [182] and human [183] genomes. In mammals, the Harvey sarcoma virus-associated oncogene is referred to as H-*ras* while the Kirsten sarcoma virus is called K-*ras* [180].

1.3.2 Ras family and structure

The mammalian *ras* family is made up of three highly conserved genes called H-*ras*, K-*ras* and N-*ras* [184]. The four guanine nucleotide binding proteins (G-proteins) encoded by the *ras* genes are known as H-Ras, K-Ras4A, K-ras4B and N-Ras, each of which is 21 kDa in size (Fig. 1.3.2, [185]) [180, 184]. The Ras proteins have a high degree of homology within their catalytic region (aa 1-164) which harbors the guanine nucleotide and effector-binding motifs [184]. The C-terminal four residues, termed the CAAX box, are conserved in all Ras proteins and are involved in posttranslational modification and membrane localization [186]. The carboxy-terminal region harboring residues 165-185 are the least conserved amongst the Ras family and is termed the

hypervariable region (HVR) [186]. It has been shown that the HVR may play a role in regulating protein-protein interactions [187].

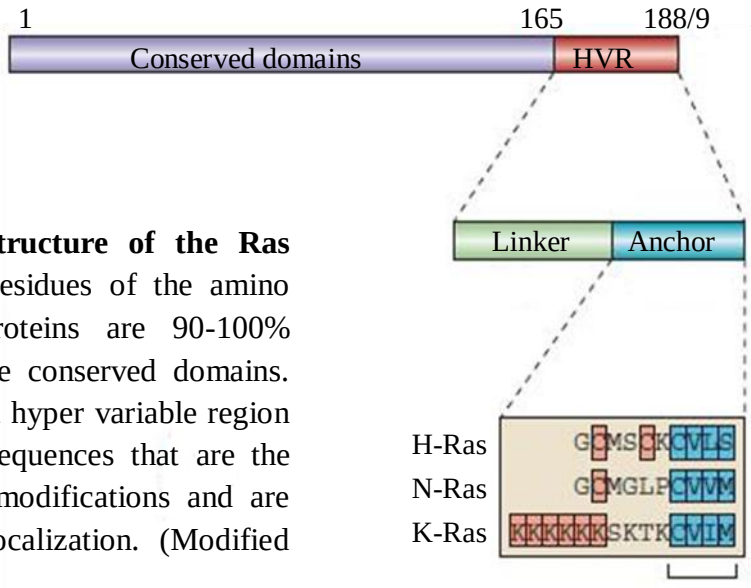

Figure 1.3.2: **Domain structure of the Ras proteins.** The first 165 residues of the amino terminal of the Ras proteins are 90-100% identical and represent the conserved domains. The significantly divergent hyper variable region (HVR) of Ras contains sequences that are the sites of posttranslational modifications and are responsible for protein localization. (Modified from [187]).

1.3.3 Ras Localization

The 'classical' Ras proteins (H-Ras, K-Ras4A, K-ras4B and N-Ras) [188] share a high degree of sequence homology but are reported to have unique biological properties. The Ras proteins carry out distinct functions as a result of their divergent subcellular localizations which are influenced by posttranslational modifications [189]. The Ras proteins are synthesized as cytosolic proteins after which farnesyltransferase catalyzes the covalent addition of a farnesyl isoprenoid lipid to the cysteine residue of the CAAX box (Fig. 1.3.3-1, [190]). Enzymes associated with the endoplasmic reticulum (ER) then proteolytically cleave the AAX residues and methylate the farnesylated cysteine residue [191]. The cysteine residues immediately upstream of the CAAX box within H-Ras and

N-Ras are palmitoylated and trafficked to the plasma membrane (PM) via the Golgi [185, 191]. In contrast, the lysine-rich sequence on K-Ras4B is responsible for the translocation of K-Ras4B from the ER to the PM [190] in a Golgi independent manner [185].

The different carboxy-terminal lipid anchors on N-Ras, H-Ras and K-Ras are reported to also target these proteins to different microdomains within the PM (Fig. 1.3.3-2, [185]). For instance, H-Ras is almost equally distributed between lipid raft and non-lipid raft regions. However, GTP binding increases the fraction of H-Ras in non-lipid raft areas [185]. On the other hand, K-Ras is predominantly associated with the non-lipid raft membrane regardless of its activation state [185].

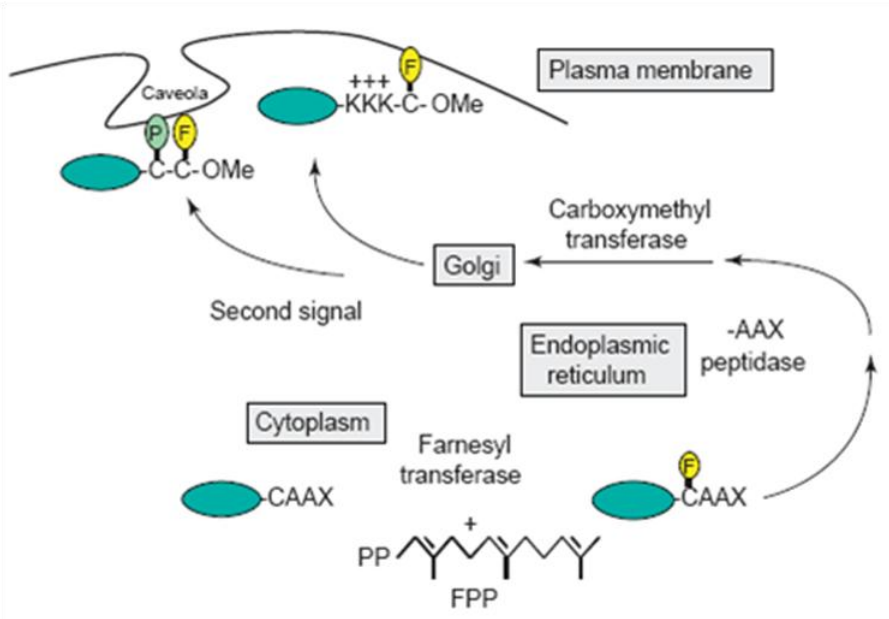

Figure 1.3.3-1: **Trafficking of Ras proteins to the plasma membrane.** Ras proteins are farnesylated in the cytoplasm. In the endoplasmic reticulum, the AAX residues are cleaved and carboxy methylationof now terminal cysteine residue occurs. The lysine residues on K-Ras4B allow it to transit directly to the plasma membrane (PM). However, H- and N-Ras are palmitoylated before they are trafficked to the PM via the Golgi apparatus [192].

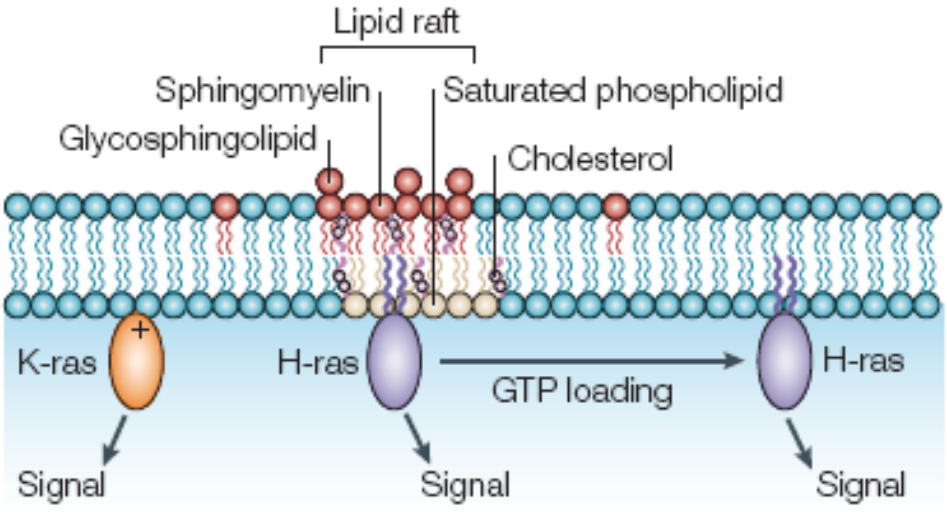

Figure 1.3.3-2: **Plasma membrane (PM) microlocalization of H- and K-Ras.** K-Ras predominantly associates with the non-lipid raft PM irrespective of its activation state. H-Ras is almost equally distributed between raft and non-raft membrane. GTP loading increases the fraction of H-Ras in the non-raft membrane [187].

1.3.4 Ras regulation

Like most GTPases, Ras functions as a molecular switch, cycling between an inactive GDP-bound state and an active GTP-bound state (Fig. 1.3.4, [192]) [193]. Extracellular signals transmitted through membrane-bound receptors stimulate guanine nucleotide exchange factors (GEFs) which promote the simultaneous dissociation of GDP and binding of the more abundant GTP to Ras [193]. This reaction is reversed by GTPase-activating proteins (GAPs) which stimulate the intrinsic Ras GTPase activity, resulting in the hydrolysis of GTP to GDP and inorganic phosphate [194]. GTP binding of Ras is induced by growth factors such as PDGF [195] and EGF [196] via activation of their cognate receptors. Ligand-induced receptor autophosphorylation subsequently stimulate the activation of son of sevenless (SOS) [197], one of the most well studied Ras

GEFs. Correspondingly, the most well studied Ras GTPase activating protein is RasGAP/p120^{GAP} [198] .

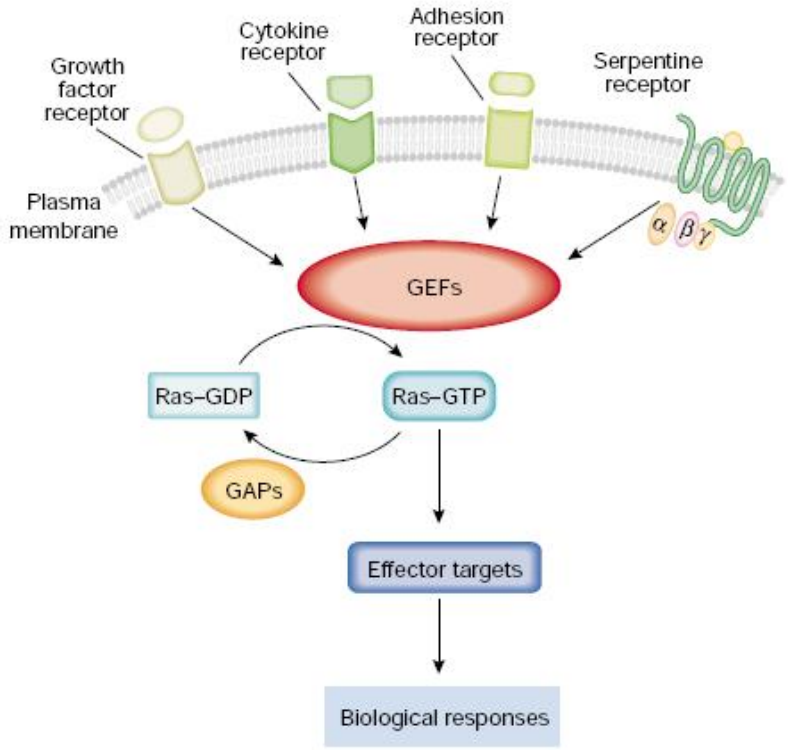

Figure 1.3.4: **Model for the cycling of activated and inactivated Ras.** Extracellular signals modulate the activity of guanine nucleotide exchange factors (GEFs) which promote GTP-binding and hence activation of Ras. Hydrolysis of GTP is promoted by GTPase-activating proteins (GAPs). Ras proteins interact with many effectors leading to diverse biological responses [194].

1.3.5 Ras signaling

Ras proteins have been shown to play a key role in signal transduction cascades by stimulating the activities of three main classes of effector proteins, Rafs, PI3-kinase (PI3K) and RalGEFs (Fig. 1.3.5, [190]), all of which become activated by binding to GTP-loaded Ras. Raf-1 is a serine-threonine kinase which was the first bona fide

mammalian Ras effector identified [199-202]. Once activated by Ras, Raf-1 then stimulates MEK1/2 which in turn activates mitogen activated protein kinases (MAPKs) such as the extracellular signal-regulated kinase 1/2 (ERK1/2) [203]. GTP-loaded Ras also binds to and activates the catalytic domain of PI3K, triggering the production of second messengers that activate the phosphoinositide-dependent kinases PDK-1 and PDK-2 which in turn activate AKT signaling pathways [203]. Ral proteins (RalA and RalB) are members of the Ras subfamily of Ras-related GTPases that also cycle between an active GTP-bound state and inactive GDP-bound state [203]. Active Ras stimulates Ral GEFs such as Ral-guanine nucleotide dissociation stimulator (GDS), which increases the activity of Ral [204, 205]. Activated Ral stimulates a variety of molecules such as PLD1 and RalBP1 [180]. Both Raf and PI3K activities are required for Ras induced transformation, as demonstrated in NIH 3T3 cells [206]. Additionally, the anti-apoptotic effects of Ras were attributed to its activation of PI3K/AKT signaling [207]. However, Hamad et al. [208] showed that the RalGEF/Ral pathway, and not the PI3K or Raf pathways, was sufficient for Ras transformation of human cells.

Another small GTPase of worthy mention is ADP-ribosylation factor (Arf), not to be confused with the tumor suppressor $p19^{Arf}$. Like other G proteins, Arfs cycle between an active GTP bound and inactive GDP-bound state and they function in processes such as membrane trafficking [209]. As it relates to Ras signaling, our lab has shown that H-Ras transformation of NIH3T3 cells possessed elevated Arf6 protein levels when compared to untransformed cells [210].

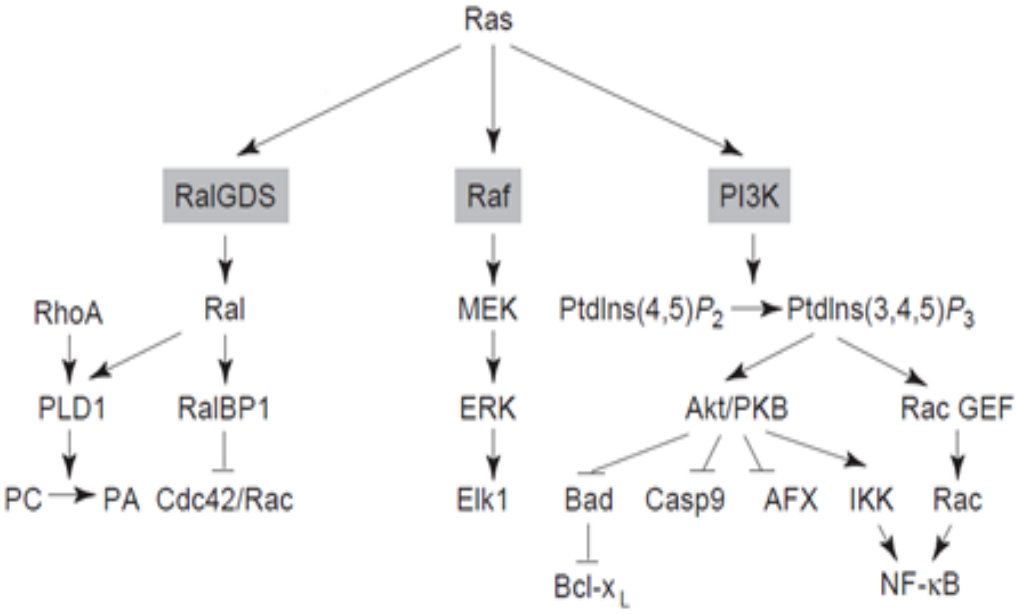

Figure 1.3.5: **Ras activates multiple effector-mediated signaling pathways.** Some of the best characterized effectors of Ras and their signaling pathways are the Raf threonine/serine kinases, phosphoinositide 3-kinases (PI3K) and the Ral GEF, RalGDS [192].

1.3.6 GTPases and PLD

Numerous reports have implicated the small GTPase family members such as Ras, Rho, Arf and RalA in the activation of PLD by various stimuli [96], some of which have been mentioned previously. Of relevance is data that showed that Ras and RalA mediate the activation of PLD in v-Src transformed cells [211, 212] and that PLD activity was required for transformation by H-RasV12 [105]. Additional reports showed that PLD activity stimulated by receptor tyrosine kinases depended on the Ras/Ral signaling cascade [64]. RalA and Arf6 also worked synergistically to elevate PLD activity in H-Ras transformed cells [210]. Additionally, our lab has shown that PLD provided a survival signal in cancer cells with activated H-Ras or K-Ras [213].

1.3.7 Ras and cancer

Activating mutations of the *ras* sequences is estimated to occur in 20-30% of all human tumors [203]. Sequence analyses revealed that there were point mutations that mostly affected residue 12 and to a lesser extent, residues 13 and 61 [214, 215]. Each of these mutations renders the GTPase domain of Ras insensitive to activation by GAP, resulting in an increase in the half-life of Ras-GTP mutants [203, 216]. The highest rate of *ras* mutations was observed in cancers of the pancreas (90%), colon (50%), lung (30%) and thyroid [203]. Particular associations were also found between the various *ras* oncogenes and certain types of human cancer [180]. For instance, pancreatic [217] and colonic [218] cancers frequently harbored mutant K-*ras* while H-*ras* mutations were predominantly found in bladder cancers [219]. N-*ras* mutations were associated mainly with lymphoid malignancies [220] and melanomas [221].

The frequency of *ras* mutations in human cancers has led to the proposal that targeting Ras signaling may be a promising strategy in the battle against cancer. As such, inhibition of Ras signaling has been shown to prevent Ras-dependent transformation and cause regression of Ras-dependent tumors in animal models [203].

1.4 AMP kinase

1.4.1 AMPK history

In order to survive, all cells must maintain a high ratio of ATP to ADP [222]. ATP synthases and adenylate kinase function to maintain the concentration of ATP at about ten times that of ADP [223]. Under conditions of cellular stress in which the rate of ATP consumption exceeds that of ATP production, there is an overall rise in the ADP:ATP ratio. As described by Hardie et al. [223], the AMP:ATP ratio vary as the square of the ADP:ATP ratio such that if the ADP:ATP ratio rises 5-fold then the AMP:ATP ratio rises 25 fold. This comparatively more dramatic change in the AMP:ATP ratio makes it a highly sensitive indicator of cellular energy status. In the late 1980s, 5'AMP-activated protein kinase (AMPK) was identified [224] and subsequent work showed that it was responsible for sensing and responding to changes in a cell's energy level.

1.4.2 AMPK structure

The heterotrimeric AMPK is a serine-threonine kinase that contains a catalytic α-subunit and regulatory β- and γ-subunits and has been described in all eukaryotes [225, 226] (Fig. 1.4.3, [227]). Each subunit has at least two isoforms so that there are twelve possible αβγ combinations of isoforms [228, 229]. The α-subunit contains several features critical for enzymatic activity and complex formation [226]. For instance, the N-terminal kinase domain contains a threonine residue (Thr-172) whose phosphorylation is essential for enzymatic activity. The C-terminal domain is required for formation of the complex with the β and γ subunits and an inhibitory domain has also been postulated to

exist immediately C-terminal to the kinase domain [230]. The β-subunit contains two highly conserved regions called the KIS and ASC domains. The KIS domain is involved in glycogen binding [231, 232] while the ASC domain is required for formation of a stable and active αβγ complex [231]. Finally, the γ-subunit contains four tandem repeats of a motif called the CBS domain whose function is to bind the adenosine portion of AMP [229].

1.4.3 AMPK localization

The β1 subunit of AMPK has been shown to be myristoylated on its N-terminus, a modification that is important for both enzymatic activity and subcellular localization. Upon overexpression of the wild-type β1 subunit in COS-7 cells, the authors found the AMPK αβγ complex primarily in membrane fractions. However, the αβγ subunits were recovered in both the soluble and membrane fractions upon overexpression of a myristoylation deficient mutant of the β1 subunit (G2A) [233]. Similarly, expression of the G2A mutant caused a shift in the localization of the β subunit from a particulate extranuclear distribution to a more homogeneous cell distribution [234]. Additional work done by Warden et al. [234] showed that introduction of phosphorylation site mutants (S24A, S25A and S182A) caused a dramatic shift towards nuclear distribution with some continued extranuclear expression.

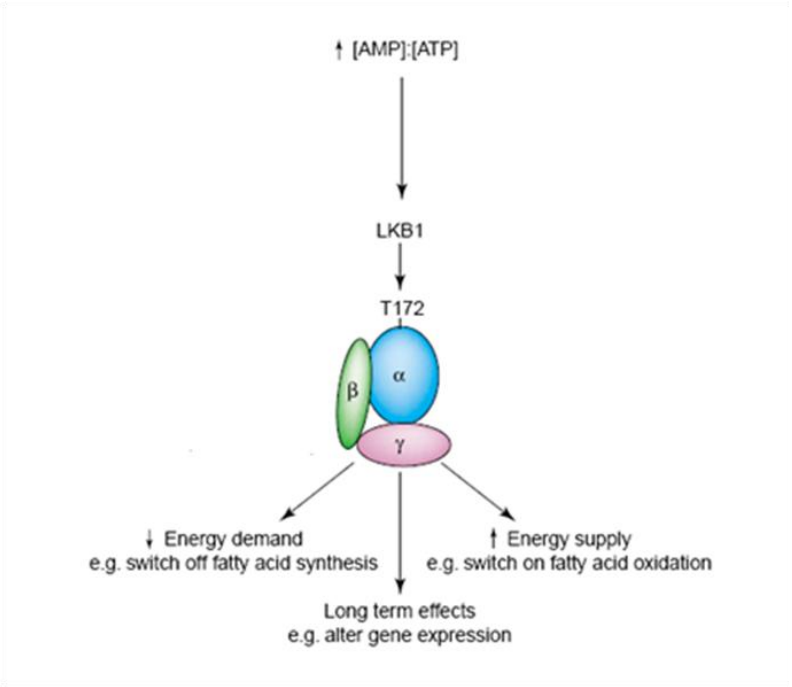

Figure 1.4.3: **5` AMP-activated protein kinase (AMPK) and function.** Conditions that cause an increase in the AMP: ATP ratio lead to activation of AMPK through increased phosphorylation on threonine 172 (T172) within the α-subunit. Once activated, AMPK phosphorylates several downstream substrates in order to turn off ATP-consuming processes and turn on ATP-generating pathways (Modified from [229].

1.4.4 AMPK regulation

The AMPK cascade is activated by any cellular stress that causes a rise in the AMP:ATP ratio due to a disruption of ATP production or consumption. Some types of stress that inhibit ATP production include, heat shock [235], oxygen deprivation (hypoxia) [236], tissue ischemia [237], glucose deprivation [238] and metabolic poisons such as arsenite or oligomycin [235]. A physiological stress that increases ATP consumption is exercise in skeletal muscle [239, 240].

AMP

Mammalian AMPK is allosterically activated by 5`-AMP (AMP), however, the heterotrimer is inactive unless phosphorylated on its threonine residue (Thr-172) by upstream kinases [241, 242]. AMPK is described as being ultrasensitive to changes in AMP given that this nucleotide activated the AMPK cascade through three mechanisms in addition to phosphorylation at Thr172. They are (i) AMP activates the upstream kinase (ii) AMP makes AMPK a better substrate for the upstream kinase and (iii) AMP makes AMPK a worse substrate for the protein phosphatase [243]. All three of these effects are antagonized by high concentrations of ATP, suggesting that both AMP and ATP compete for binding to the allosteric site. This competitive action of the adenosine nucleotides also imply that AMPK is activated by a simultaneous increase in AMP and decrease in ATP levels [226].

Upstream kinases

Phosphorylation of AMPK at Thr172 is critical for enzymatic activity, and the kinases responsible for this phosphorylation have been identified as LKB1 and possibly Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) [244]. LKB1 is a tumor suppressor gene that is lost in Peutz-Jeghers syndrome [245]. Individuals with this condition are at a higher risk of developing malignant tumors including of the breast [246, 247] and lung [248, 249]. LKB1 is the primary kinase that activates AMPK through phosphorylation of Thr172 [250-252] and is essential for the activation of AMPK by exercise [253]or the anti-diabetic drug metformin [244]. In mammalian cells that lack LKB1 expression, AMPK could not be activated by another anti-diabetic drug

phenformin or an AMP mimetic, 5-aminoimidazole 4-carboxamide riboside (AICAR) [250]. Recently, AMPK was shown to be activated by a Ca^{2+}-dependent but AMP-independent mechanism which is dependent on CaMKK [254]. An increase in AMPK associated with CaMKK was also observed when AMPK activity was stimulated in a CaMKK dependent manner [255].

AMPK inhibitors

Phosphocreatine (PCr) is a high energy phosphate storage molecule that serves as a temporary energy buffer during contraction in skeletal muscle where ATP is used up faster than it is produced [256]. PCr donates a phosphate group to ADP in a reversible reaction catalyzed by creatine kinase [257]. In addition to regulation by the ATP:AMP ratio, Ponticos et al. [258] showed that AMPK activity is modulated by the phosphocreatine:creatine (PCr:Cr) ratio and that AMPK is allosterically inhibited by PCr. Glycogen is another source of stored energy in cells and the AMPK β subunit contains a glycogen binding domain [231, 232]. In studies of rat and human muscle, elevated glycogen levels suppress AMPK activity, suggesting that AMPK may also function as a sensor of glycogen content [259, 260].

1.4.5 AMPK signaling

The actions of AMPK have been best studied in exercising skeletal muscle or in cells treated with AMPK activators such as AICAR. When taken up by cells, AICAR is phosphorylated by adenosine kinase to produce the monophosphorylated derivative ZMP. This derivative mimics all four effects of AMP on the AMPK cascade without perturbing the levels of ATP, ADP or AMP [261].

Proteins inhibited by AMPK

Upon activation, AMPK inhibits a number of key metabolic enzymes that consume ATP but are not immediately essential for survival (Fig. 1.4.5, [226]). These proteins include mammalian target of rapamycin (mTOR) [262], acetyl-CoA carboxylase 1 (ACC1) [226], fatty acid synthase (FAS) [263] and HMG-CoA reductase [264], key regulators of protein, fatty acid and cholesterol/isoprenoid biosynthesis, respectively. Another action of AMPK is to activate catabolic processes that produce ATP. For instance, AMPK activation acutely stimulates glucose uptake via the GLUT1 and GLUT4 receptors, fatty acid oxidation by inhibiting ACC2, and promotes glycolysis through the activation of phosphofructose kinase 2 (PFK2) [226]. The expression of numerous genes is also increased by AMPK and includes GLUT1 and GLUT4 [265], hexokinase II [265] and mitochondrial enzymes involved in the TCA cycle and respiratory chain in muscle [223].

Of interest is mTOR, a serine-threonine kinase that plays a central role in the regulation of growth and proliferation by controlling protein synthesis [266]. mTOR is regulated by growth factors and nutrients and is part of two distinct multiprotein complexes. mTORC1 is sensitive to rapamycin while mTORC2 is insensitive to rapamycin [267]. Growth factors such as insulin and nutrients such as amino acids and glucose stimulate mTORC1 activity as demonstrated by increased phosphorylation of two key regulators of protein translation: ribosomal p70S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1) [267]. The key role of mTORC2, on the other hand, is to phosphorylate Akt at its Ser473 site [244]. Work by Krause et al. showed that treatment of isolated hepatocytes with AICAR, blocked the amino acid-

induced activation of p70S6K [268]. The mechanism of AMPK inhibition of mTOR appears to be indirect as Inoki et al. [269] demonstrated that AMPK phosphorylates and activates TSC2, a tumor suppressor which subsequently inhibits mTOR. More recently, it has been reported that AMPK inhibits mTORC1 by phosphorylating and inhibiting raptor, a component of mTOR complex 1 [270].

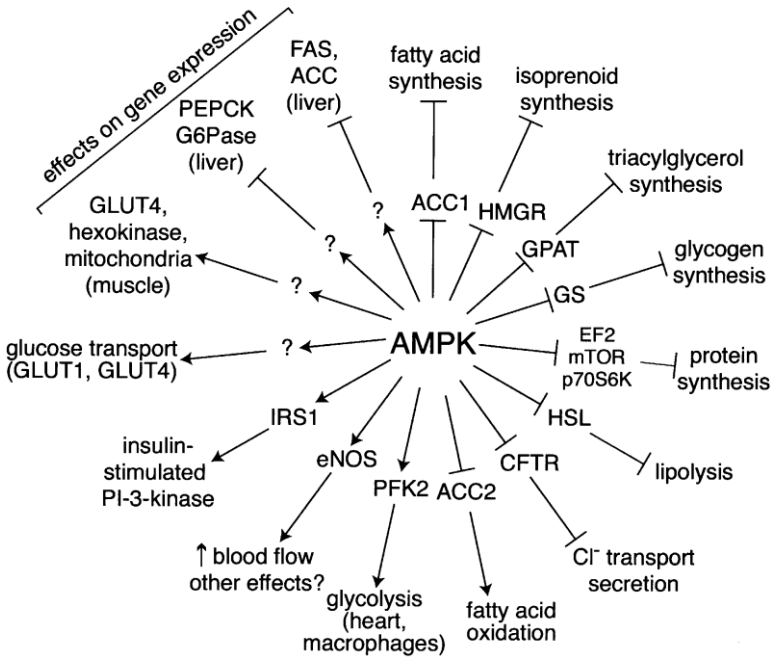

Figure 1.4.5: **Proteins and pathways regulated by AMPK.** AMPK suppresses the activity of several proteins involved in key energy consuming processes such as isoprenoid, fatty acid and protein synthesis. ATP-generating processes such as fatty acid oxidation and glycolysis [228].

1.4.6 AMPK in disease

Diabetes

Type 2 diabetes is characterized by abnormally high blood glucose levels as a result of insulin resistance and is often managed by diet and exercise. As a result of a

correlation between increased exercise and elevated AMPK activity [239, 240], many speculated that the metabolic abnormalities associated with type 2 diabetes could be reversed by activation of AMPK [271]. Using animal models of type 2 diabetes, researchers have shown that continual administration of AICAR reduced plasma triglycerides, fatty acids, endogenous glucose production [272], lowered the systolic blood pressure [273] and reduced abdominal fat [274]. The antidiabetic drugs metformin and rosiglitazone have been shown to exert their effects through activation of the AMPK pathway [275]. These observations may explain the beneficial results of physical exercise on patients with type 2 diabetes [276].

Cancer

The study of the role of AMPK in cancer is still an emerging field but the investigations so far have produced contradictory, yet intriguing results. Specifically, the paradox involves reports that show that AMPK has anti-apoptotic and pro-apoptotic effects. For instance, because AMPK has inhibitory effects on ACC, FAS and mTOR, it was believed that activation of AMPK may selectively cripple the growth or survival of cancer cells that depend on these proteins [277]. Treatment of the prostate cancer cell lines PC3, Du145, and LNCaP with AICAR inhibited their growth and survival [278]. Others have obtained similar results supporting the hypothesis that elevating AMPK activity leads to apoptosis in cancer cell lines [279, 280]. In contrast, elevated AMPK activity has been shown to protect both normal and cancerous cells against death induced by various stresses. For instance, stimulation of AMPK activity prevented fatty acid-induced apoptosis in astrocytes [281] and AICAR inhibited apoptosis in fibroblasts deprived of growth factors [282]. Importantly, a report by Kato et al. demonstrate that

pancreatic cancer cells depend on AMPK signaling in order to survive nutrient-deprived conditions [283]. Although in its infancy, the data collected so far suggests that targeting AMPK may prove crucial in eradicating cancers that depend on AMPK signaling for survival.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Solutions and reagents

DMEM, bovine calf serum, lipofectamine 2000, lipofectamine LTX and RNAi MAX were obtained from Invitrogen. Antibiotic-antimycodic solution (10,000 units/ml penicillin G, 10 mg/ml streptomycin sulfate and 25µg/ml amphotericin B), DMSO, 1-butanol, EGF, actin and β-tubulin antibodies were from Sigma. ^{3}H-myristic acid, Econofluor-2 scintillation fluid, En^{3}Hancer Spray were purchased from Perkin Elmer. 100X Protease Inhibitor Cocktail Set I, 100X Phosphatase Inhibitor Cocktail Set I, AG1478 and Compound C were from Calbiochem. AICAR and the RalA activation assay kit were from Upstate. The Ras activation assay kit was from Pierce and the Silica gel G, 60A TLC plates were from Fisher. The BioCoat^{TM} invasion chambers were from BD Biosciences. Diff-Quik staining kit was from dade Behring and honokiol was a gift from Jack Arbiser.

2.1.2 Cells lines

MDA-MB-231, MDA-MB-468, MCF7, Calu-1 and T24 cell lines were obtained from American Type Culture Collection (ATCC).

2.1.3 Plasmids and siRNA

Vectors used were pcDNA3.1(-) (Invitrogen) and pcDNA3.1(-)-RasN17, which was constructed by inserting the S17N Ras gene from pCMV-RasN17 (Clontech) using flanking *Eco*R I and *Bam*H I sites. pcDNA3.1(-)ARF1T31N, pcDNA3.1(-)ARF6T27N were described previously [284] and pcDNA3.1(-)RalAS28N was also described

previously [285]. Scrambled siRNA and siRNA targeting H-Ras, K-Ras, LKB1 and AMPK were obtained from Sigma. EGFR siRNA was from Upstate.

2.1.4 Antibodies

Antibodies against EGFR, HER2 and phosphorylated EGFR (Tyr1173) were from Upstate. AMPKα and phosphorylated AMPKα (Thr172), p70S6K and phosphorylated p70S6K (Thr389), AKT and phosphorylated AKT (Ser473), PARP, PLD1, p42/44 MAPK and phosphorylated p42/44 MAPK (Thr202/Tyr204) antibodies were from Cell Signaling. H-Ras, K-Ras and GLUT4 antibodies were from Santa Cruz. PLD2 antibody was from Novus Biologicals. Secondary mouse and rabbit antibodies were from Promega.

2.2 Methods

2.2.1 Cell culture conditions and transfections

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum. The cells were incubated in a humidified chamber at 37ºC and 5% CO_2. Cells were seeded into 60-mm tissue culture plates at a density that would allow them to be 30-50% confluent overnight. Transfection of siRNAs was performed using RNAi MAX according to the manufacturer's instructions. For transfections of plasmid DNA, cells were seeded at a density that would allow them to be 80% confluent overnight. Transfections were performed using Lipofectamine 2000 or Lipofectamine LTX according to the manufacturer's instructions.

2.2.2 Immunoblotting

Cells were washed twice in ice-cold 1X PBS and residual PBS was aspirated. An appropriate volume of 1% modified Ripa buffer (65 mM Tris-HCl (pH7.4), 154 mM NaCl, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM EDTA, 1mM activated sodium orthovanadate, 1 mM NaF, 1X Protease Inhibitor Cocktail Set I, 1X Phosphatase Inhibitor Cocktail Set I) was added to the plates and cells were scraped and collected in cold micro-centrifuge tubes. These tubes were then rotated at 4ºC for 25 minutes and centrifuged for 10 minutes at 13,000 rpm and 4ºC. The supernatant was removed and placed into clean ice-cold micro-centrifuge tubes. Protein concentrations were determined using the Bio-Rad DC protein assay and equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked and probed with antibodies in accordance with the manufacturers' instructions.

2.2.3 PLD assay

The media of 90% confluent cells was switched to the appropriate serum concentration and incubated overnight. Cells were labeled with ^{3}H-myristic acid (3.0 µCi) for 4-6 hrs and 1-butanol (0.8%) was added 20 minutes before lipids were collected. Cells were washed twice in ice-cold 1X PBS (pH7.4) after which 500 µl of ice-cold methanol: 6N HCl (50:2) was added. The cells were scrapped and placed into the first extraction tube (155 µl 1N NaCl, 500 µl ice-cold chloroform) which was vortexed for 30 seconds and centrifuged at 4°C for 3 minutes at 13,000 rpm. The upper aqueous layer was discarded and the lower organic phase was transferred to the second extraction tube (350 µl water, 115 µl methanol, 115 µl 1N NaCl) which was vortexed for 15 seconds and centrifuged as

above. Most of the upper aqueous phase was removed, leaving just enough to cover the lower organic phase.

The experiment could be stopped here and samples stored at -80°C. The CPM for each sample was measured by transferring 10 µl of the lower organic phase into 3 ml of Econofluor-2 scintillation fluid. The CPMs were normalized and 5-7 x 10^5 CPM of each sample was dried with nitrogen gas for 10 minutes. The samples were resuspended in 30 µl of spotting solution (chloroform: methanol, 9:1), vortexed briefly and centrifuged briefly. The samples were spotted (10 µl at a time) onto a Silica gel G, 60A TLC plate and placed in a chamber containing 100 ml of a mobile phase solution (ethyl acetate: iso-Octane: glacial acetic acid: water, 88:40:16:80) for 2 hrs. The plate was allowed to air dry for 20 min, sprayed with En^{3}hancer and transferred to a polyethylene bag. The plate was then placed in an exposing cassette with film and developed after 3-5 days at -80°C.

2.2.4 Ras assay

Lysates were collected and protein concentration was determined as in Materials and Methods (2.2.2). 20 µg of protein was used for the total lysate control while larger amounts of protein (0.5-1 mg) were subjected to the Ras assay according to the manufacturer's instructions. In principle, the EZ-detect Ras Activation assay kit uses a GST-fusion protein containing the Ras-binding domain (RBD) of Raf1 to specifically pull-down active Ras. The kit then uses an anti-Ras antibody to detect the purified Ras by immunoblotting. GST-Raf1-RBD is incubated with cell lysate in the presence of glutathione agarose beads. The active Ras that binds to the GST-Raf1-RBD, which in turn binds to the glutathione agarose beads, is then detected by immunoblotting using

anti-Ras antibody. Because only the active form of Ras can bind to Raf1-RBD, only the active Ras is purified and detected during western blotting.

2.2.5 RalA assay

Lysates were collected and protein concentration was determined as in Materials and Methods (2.2.2). 20 µg of protein was used for the total lysate control while larger amounts of protein (1 mg) were subjected to the Ral assay according to the manufacturer's instructions. In principle, the RalA activation assay kit uses a GST fusion-protein corresponding to residues 397-519 of Ral binding protein (RalBP1) bound to agarose. RalBP1 specifically binds to and precipitates Ral-GTP from cell lysates which is then detected using an anti-RalA antibody supplied with the kit.

2.2.6 Migration assay

The assays were carried out using BIOCOAT^{TM} cell culture inserts that had polyethylene terephthalate filters (8 µm pore size) on the bottom. For migration assays, inserts were used directly without coating. Single cell suspensions in 10% or 0.5% serum concentrations were placed into the inserts. The inserts were set into 24-well plates that held 0.75 ml/well of 10% serum growth medium and incubated at 37°C and 5% CO_2 for 24 hrs. Cells that had not penetrated the filters were wiped out with cotton swabs, and cells that had migrated to the lower surface of the filters were fixed and stained using the Diff-Quik staining kit according to the manufacturer's instructions. The number of migrated cells was counted under a microscope. The mean of five individual fields in the center of the filter where migration was the highest was obtained for each well.

CHAPTER III

PLD ACTIVITY INDUCED BY SERUM-WITHDRAWAL DEPENDS ON EGFR, RAS, RALA, ARF1AND ARF6

3.1 Introduction

Our lab has shown that various cancer cell lines such as MDA-MB-231, are able to survive in media containing as little as 0.5% serum. Exposure of these cells to such serum deprived conditions caused a rapid increase in PLD activity which was observed within 15 minutes [130]. We hypothesized that under the stress of serum withdrawal, these cells may be generating anti-apoptotic signals, perhaps mediated by PLD, in order to survive. In support of this idea, Zhong et al. [113] demonstrated that suppression of PLD activity induced apoptosis of MDA-MB-231 cells deprived of serum. Additionally, depriving these cells of serum also promoted cell migration and invasion, functions that were both dependent on PLD [130].

Historically, PLD activity has been shown to be stimulated by molecules such as growth factors, which are found in abundance in serum. Therefore, these results in which PLD activity was induced by a lack of serum, and therefore, a lack of growth factors, indicated that PLD may be generating "survival signals" for certain cancer cells exposed to low serum conditions. Although PLD activity was increased, the PLD1 and PLD2 protein levels remained unchanged, suggesting that the induction of PLD activity occurred at the level of regulation but not expression. We hypothesized that the PLD activity induced by serum withdrawal occurred through two possible means: (i) serum contained inhibitory factor(s) thus withdrawing serum relieves the inhibition on PLD activity or (ii) cells sense the decrease in serum and activate signaling pathways responsible for the stimulation of PLD activity. In order to address the latter possibility, the goal of my work was to decipher the upstream signaling pathways that contribute to the induction of PLD activity under serum deprived conditions.

To that end, we focused our attention on proteins such as EGFR and Ras, which have been previously implicated in PLD activation (albeit in conditions in which growth factors were added to stimulate PLD activity). EGFR signaling can be amplified due to overexpression, mutation or autocrine growth factors loops [167]. Likewise, Ras signaling can be augmented by activating mutations that render the GTP-bound Ras insensitive to the actions GAPs [203]. Our rationale for studying EGFR and Ras is based on evidence that the cancer cell lines that display elevated PLD activity are also reported to express elevated levels of EGFR [286] and contain activating mutations in either K-*ras* [287, 288] or H-*ras* [215]. In this chapter, we report that EGFR, Ras and downstream G-proteins all play a role in regulating the induction of PLD activity when cancer cells are maintained in serum deprived conditions.

3.2 Results

3.2.1 No correlation between the amount of EGFR and level of PLD activity

Based on a report by Moasser et al. [289] and observations made in our lab, we hypothesized that if EGFR expression correlated with PLD activity, then the cell line with the highest EGFR expression will also exhibit the highest PLD activity. To test this idea, we compared the PLD activity of three breast cancer cell lines, MCF7, MDA-MB-231 and MDA-MB-468 which express varying levels of EGFR. Correlating with published reports [289, 290], the MDA-MB-468 cells expressed the highest levels of EGFR and undetectable levels of HER2 (Fig. 3.2.1A). The MDA-MB-231 cells displayed moderate levels of both EGFR and HER2 while only HER2 was detected in the MCF7 cells. Comparison of the PLD activities of these cell lines showed that while the MCF7 cells had the lowest PLD activity, the MDA-MB-231 and the MDA-MB-468 cells had comparable PLD activities as revealed by phosphatidylbutanol (PBt) production (Fig. 3.2.1B). These results did not support our hypothesis since there was no correlation between the amount of EGFR and the level of PLD activity. However, the cell lines with the highest PLD activity both expressed EGFR, suggesting that EGFR expression may be necessary but not sufficient for the elevated PLD activity.

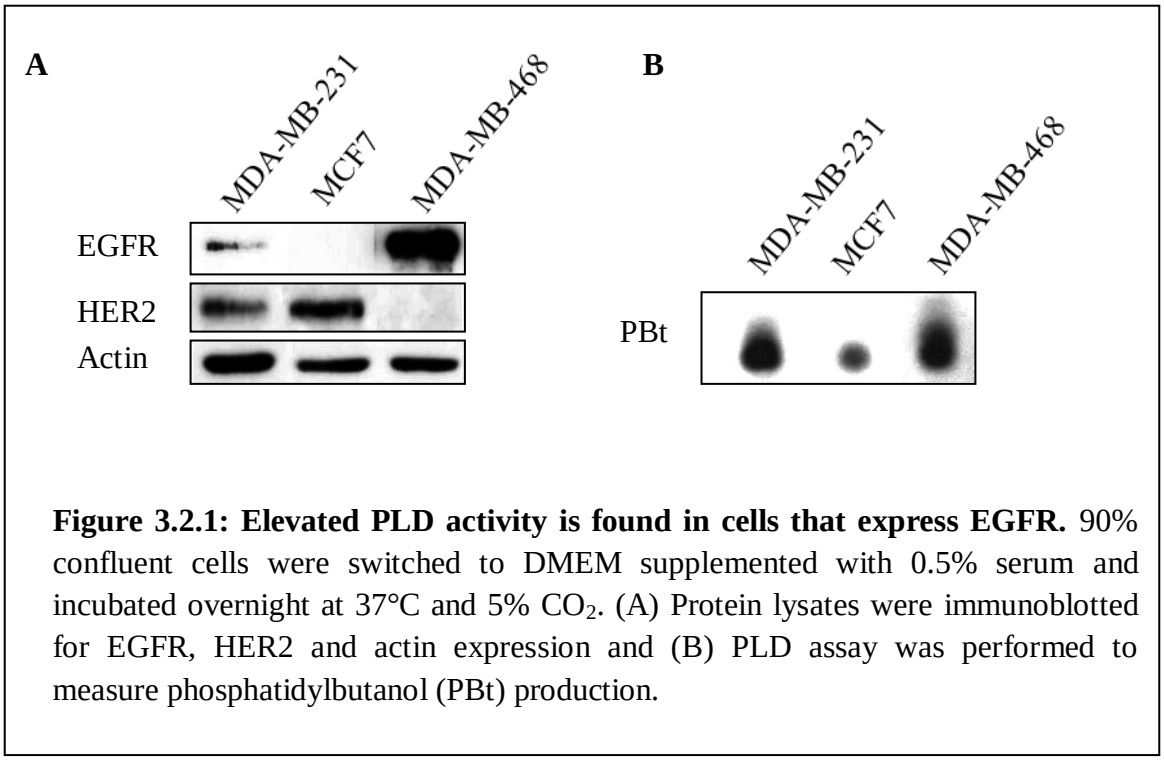

Figure 3.2.1: Elevated PLD activity is found in cells that express EGFR. 90% confluent cells were switched to DMEM supplemented with 0.5% serum and incubated overnight at 37°C and 5% CO_2. (A) Protein lysates were immunoblotted for EGFR, HER2 and actin expression and (B) PLD assay was performed to measure phosphatidylbutanol (PBt) production.

3.2.2 Correlation between EGFR activation and expression with PLD activity

The above data indicated a potential relationship between PLD activity and EGFR. To test this idea, we examined the expression and activation of EGFR in MDA-MB-231 cells deprived of serum. Since the withdrawal of serum induces PLD activity in the aforementioned cells, we hypothesized that activation and/or expression of EGFR may be similarly affected. Indeed, maintenance of MDA-MB-231 cells in serum deprived conditions overnight resulted in a modest increase in the activation of EGFR as indicated by the phosphorylation of EGFR at tyrosine 1173 (Y1173) (Fig. 3.2.2A). Additionally, we observed a dramatic increase in the expression of EGFR which correlates with the increase in PLD activity observed in 0.5% serum (Fig. 3.2.2B). The protein levels of PLD1 and PLD2 however, remain unchanged (Fig. 3.2.2C).

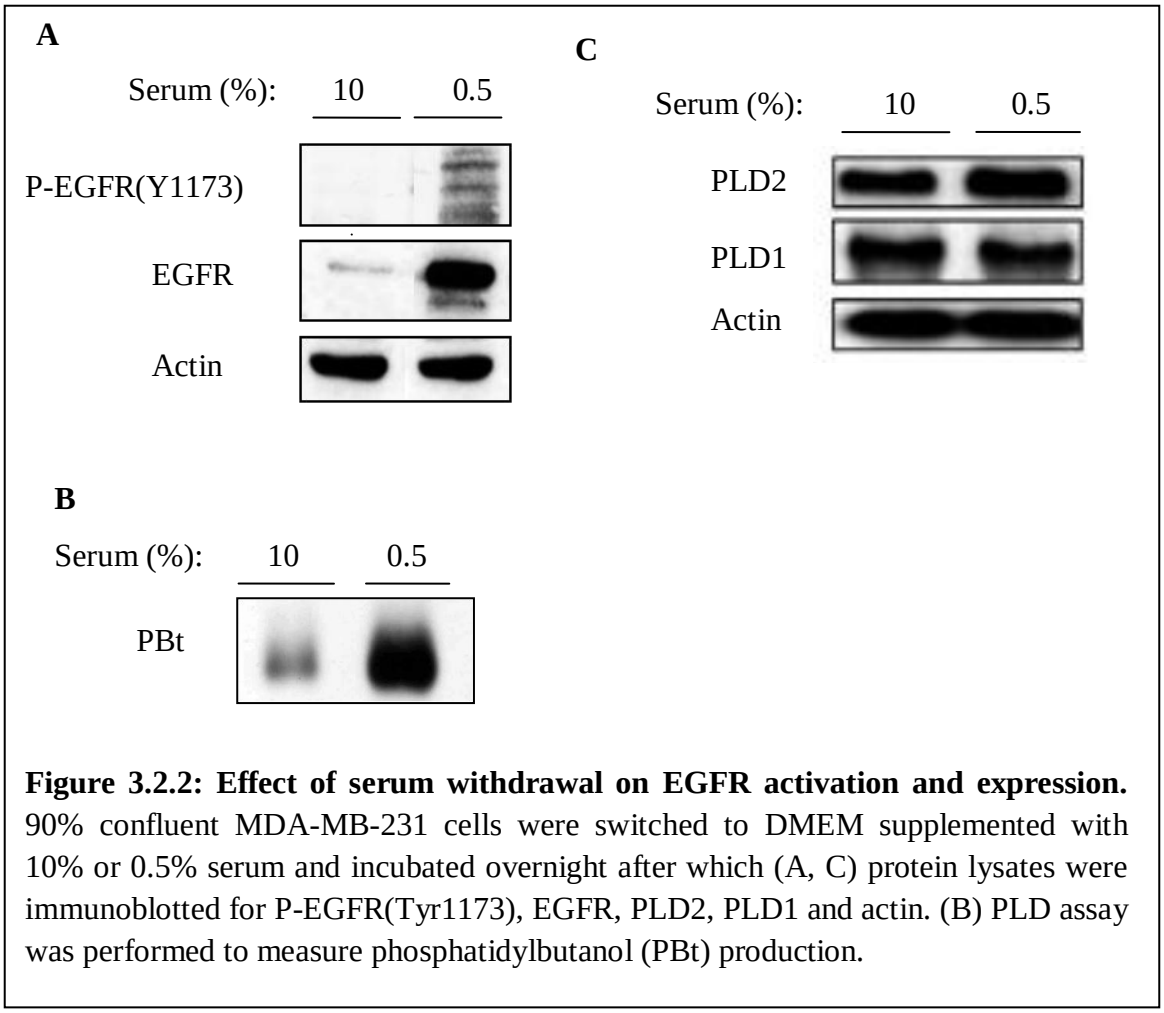

Figure 3.2.2: Effect of serum withdrawal on EGFR activation and expression. 90% confluent MDA-MB-231 cells were switched to DMEM supplemented with 10% or 0.5% serum and incubated overnight after which (A, C) protein lysates were immunoblotted for P-EGFR(Tyr1173), EGFR, PLD2, PLD1 and actin. (B) PLD assay was performed to measure phosphatidylbutanol (PBt) production.

3.2.3 Elevated PLD activity depends on EGFR

In keeping with the goal of determining factors and pathways involved in the stimulation of PLD activity under stress, we wanted to ascertain whether the induction of PLD activity required signals emanating from EGFR. To that end, we utilized pharmacological inhibitors and siRNA techniques to test our hypothesis that suppression of EGFR would reduce stress-induced PLD activity.

Our initial approach to inhibiting EGFR involved the use of AG1478, a highly potent and specific inhibitor that functions as an ATP competitor with an IC_{50} of 3 nM

(Fig. 3.2.3-1A). Significantly higher concentrations (> 100 μM) of AG1478 are required for the inhibition of other receptors such as HER2 and the PDGF receptor. Thus, applying AG1478 at a concentration of 250 nM was not expected to non-specifically affect the activities of other proteins. In order to confirm that use of AG1478 inhibited EGFR signaling pathways we examined the effects of 250 nM AG1478 on EGF stimulated Ras and MAPK activities. Treatment of MDA-MB-231 cells with 100 ng/ml EGF caused an increase in GTP bound Ras and phosphorylation of p44/p42 MAPK (Fig. 3.2.3-1B). However, pre-treatment (40 min) of cells with 250 nM AG1478 reduced these effects, indicating that AG1478 was effective at inhibiting signals stimulated by EGFR activation.

As shown in Fig. 3.2.3-1C, treatment of MDA-MB-231 cells with AG1478 significantly suppressed the PLD activity in 0.5% serum with little effect on the PBt production in 10% serum. This result indicated that the PLD activity induced by serum withdrawal depends on the activation of EGFR.

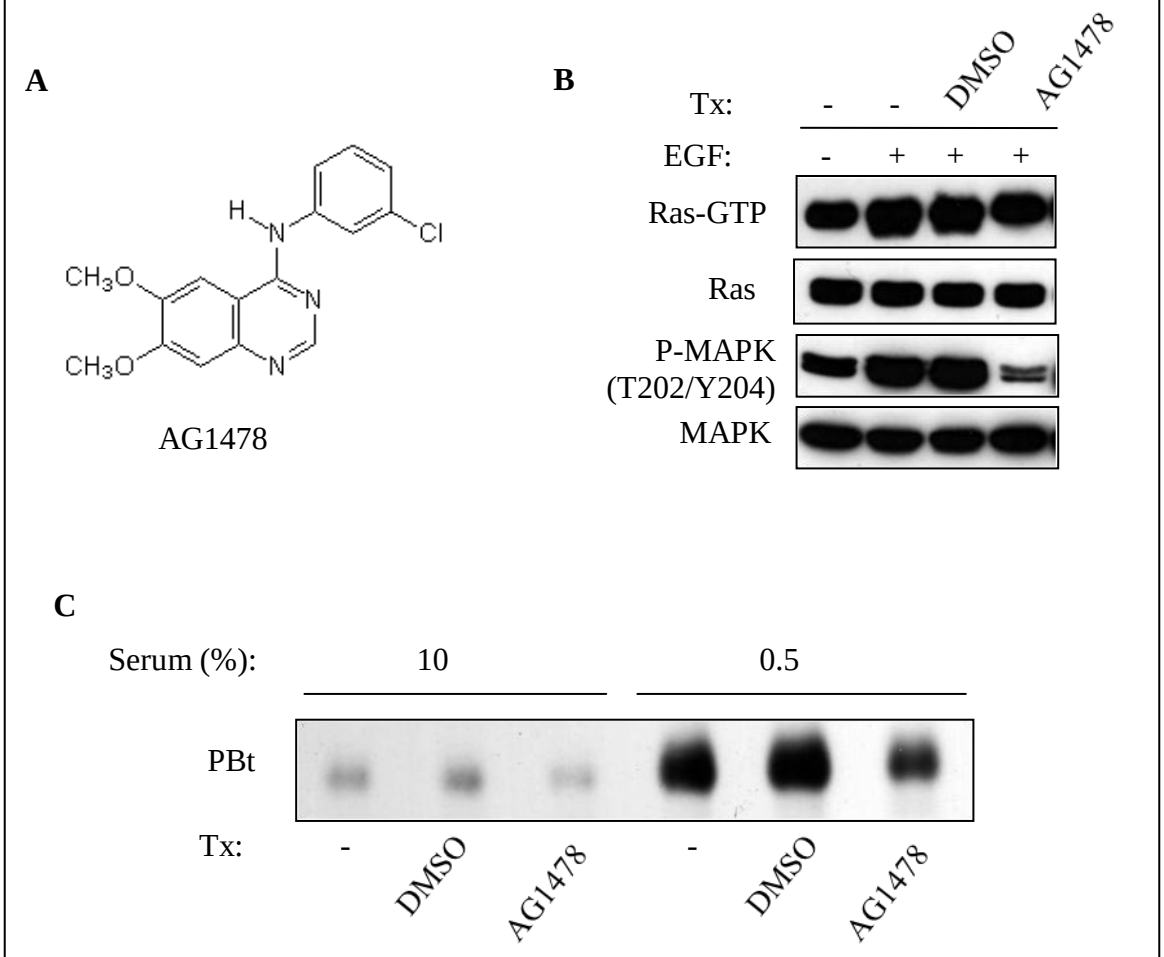

Figure 3.2.3-1: Pharmacological inhibition of EGFR suppresses PLD activity. (A) Structure of the EGFR inhibitor AG1478. (B) 90% confluent MDA-MB-231 cells were switched to DMEM supplemented with 0.5% serum and incubated overnight. Cells were pre-treated with DMSO or 250 nM AG1478 for 40 min and treated with EGF (100 ng/ml) for 10 min. Protein lysates were used to detect active Ras (Ras-GTP) and immunoblotted for total Ras, P-MAPK(Thr202/Tyr204) and MAPK. (C) 90% confluent MDA-MB-231 cells were switched to DMEM supplemented with 10% or 0.5% serum and incubated overnight. Cells were pre-treated with DMSO or 250 nM AG1478 40 min before lipids were collected and phosphatidylbutanol (PBt) production was measured.

To further establish the requirement for EGFR in the stimulation of PLD activity under stress, we used siRNA to knockdown the expression of EGFR and assayed its effects on PLD activity. The siRNA targeted knockdown of EGFR was very efficient since there was approximately a 90% reduction in EGFR protein levels (Fig. 3.2.3-2A). As expected, reduction of EGFR expression also reduced the PLD activity induced by serum withdrawal (Fig. 3.2.3-2B). The results presented here clearly implicate a role for EGFR in the stimulation of PLD activity despite the lack of serum and supports our hypothesis that suppression of EGFR would inhibit this stress-induced PLD activity.

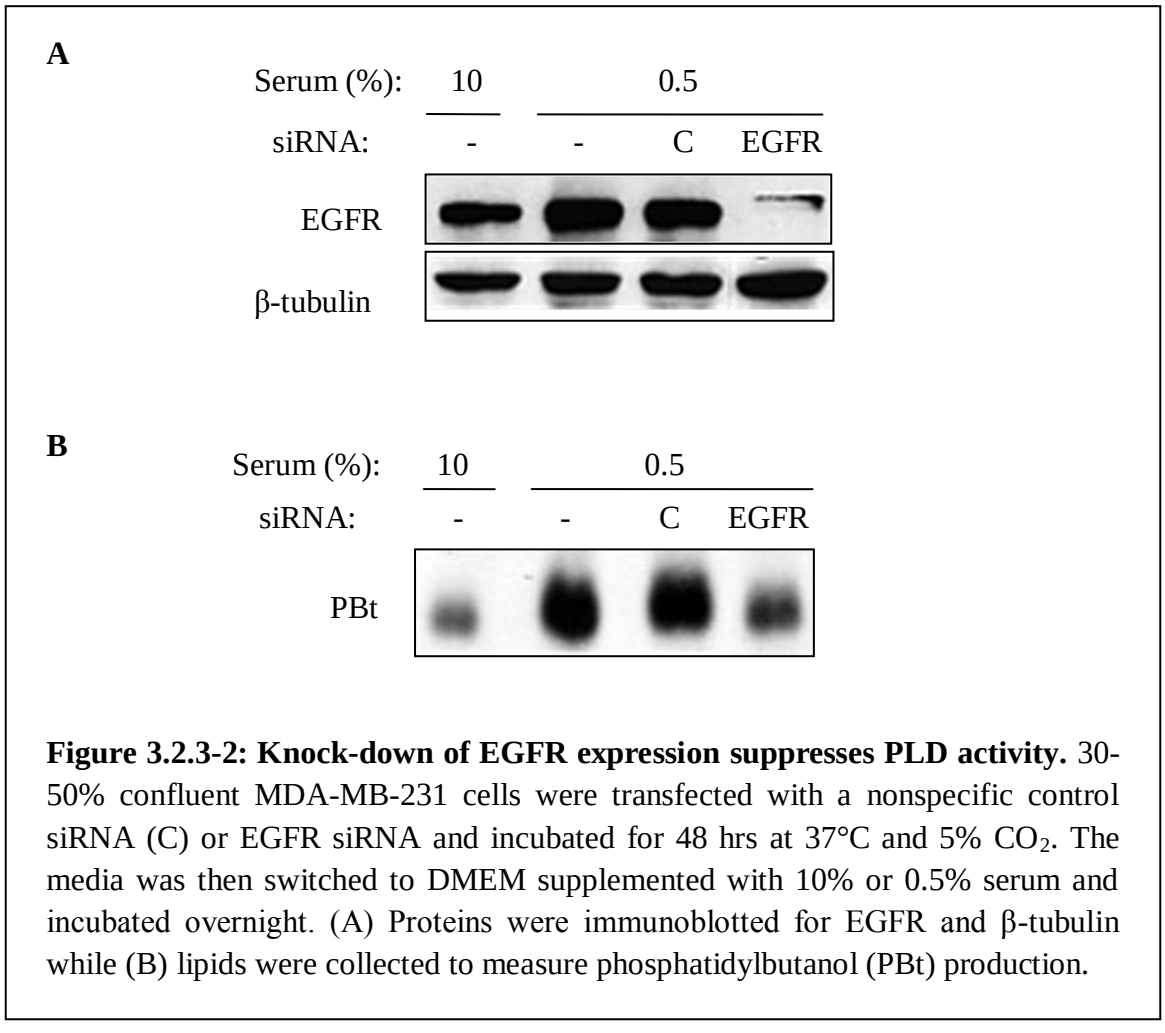

Figure 3.2.3-2: Knock-down of EGFR expression suppresses PLD activity. 30-50% confluent MDA-MB-231 cells were transfected with a nonspecific control siRNA (C) or EGFR siRNA and incubated for 48 hrs at 37°C and 5% CO_2. The media was then switched to DMEM supplemented with 10% or 0.5% serum and incubated overnight. (A) Proteins were immunoblotted for EGFR and β-tubulin while (B) lipids were collected to measure phosphatidylbutanol (PBt) production.

3.2.4 Correlation between Ras activity and PLD activity in low serum

Another potential player in the regulation of PLD activity in low serum is Ras, a GTPase that plays a key role in signal transduction cascades that originate from receptors such as EGFR. Ras has been implicated in the activation of PLD by various stimuli [96] and transforming oncogenes [105, 211]. Of significance is the fact that the MDA-MB-231 breast cancer cell line harbors a mutation in its K-*ras* sequence [287]. In one of the K-*ras* alleles, the authors reported the presence of an adenosine instead of a guanosine in the second position of codon 13. The resulting mutant has an aspartic acid at codon 13 instead of glycine. As mentioned previously, activating mutants of the Ras proteins render them insensitive to GAP action, hence prolonging the life span of the active form of Ras.

Active GTP-bound Ras binds to numerous effectors by associating with their Ras binding domains (RBD). The Ras assay used in this work employs the Raf1 RBD to "pull-down" GTP bound Ras from cell lysates. In order to address the question of whether Ras plays a role in the regulation of PLD activity in the MDA-MB-231 cells, we first examined the effect of serum withdrawal on Ras activity. As shown in Fig. 3.2.4A, MDA-MB-231 cells maintained overnight in 0.5% serum conditions have higher amounts of GTP-bound Ras than cells in 10% serum. To determine how quickly Ras activity increased, we assayed for active Ras after cells were exposed to low serum conditions for 1 and 4 hours. The results show that the increase in activated Ras could be observed within 1 hour of serum deprivation (Fig. 3.2.4B). This correlates with our published report in which PLD activity was elevated after 1 hr in low serum conditions [130].

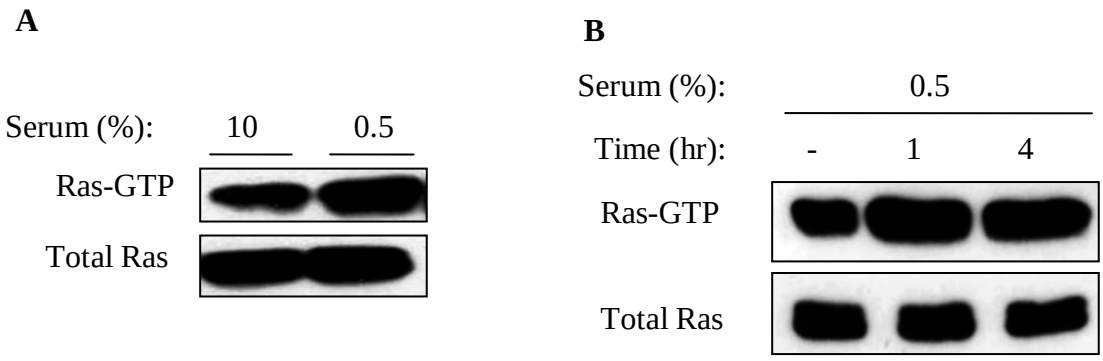

Figure 3.2.4: Effect of serum withdrawal on Ras activity. 90% confluent MDA-MB-231 cells were switched to DMEM supplemented with 10 % or 0.5% serum where indicated and (A) incubated overnight or (B) for 1 and 4 hrs after which the levels of GTP bound and total Ras was determined

3.2.5 Elevated PLD activity depends on Ras

The observation that Ras activity is also induced by serum withdrawal led us to hypothesize that the PLD activity may also be dependent on Ras activity. Our first approach involved the use of a dominant negative mutant of H-Ras in which there is a substitution of asparagine for serine at position 17 (RasN17). Transfection of MDA-MB-231 with RasN17 abrogated the induction of PLD activity in low serum when compared to the control vector (Fig. 3.2.5-1). One of the caveats however, of using the RasN17 mutant is that it is nonspecific since it functions by forming a nonproductive complex with Ras GEFs, hence affecting the activities of not only H-, K-, N-Ras but also the activities of proteins that share GEFs with Ras [190, 291]. As a result, we turned to siRNA technology to specifically and efficiently reduce the activity of Ras.

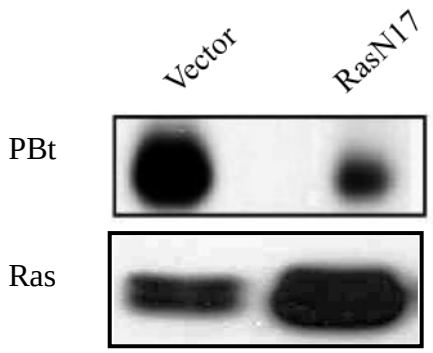

Figure 3.2.5-1: Suppression of Ras activity by RasN17 reduces PLD activity. 80% confluent MDA-MB-231 cells were transfected with an empty vector or a vector harboring the RasN17 mutant and incubated overnight. The media was then switched to DMEM supplemented with 0.5% serum after which (top panel) phosphatidylbutanol (PBt) production was determined and (bottom panel) lysates were immunoblotted for Ras

As mentioned previously, the MDA-MB-231 cell line harbors an activating mutation in one allele for K-*ras* but harbors wild-type H-*ras*. We used siRNA to specifically knock down the expression of both Ras proteins. A 100% reduction in K-Ras protein levels (Fig. 3.2.5-2A) caused a dramatic decrease in PLD activity (Fig.3.2.5-2B). Likewise, the inhibition of H-Ras, as demonstrated by the reduction in the phosphorylation levels of p44/p42 MAPK (Fig. 3.2.5-2C), resulted in a similar decrease in PLD activity (Fig. 3.2.5-2D). Taken together, this data indicates that the PLD activity of the MDA-MB-231 cells depend on both wild-type and mutant Ras.

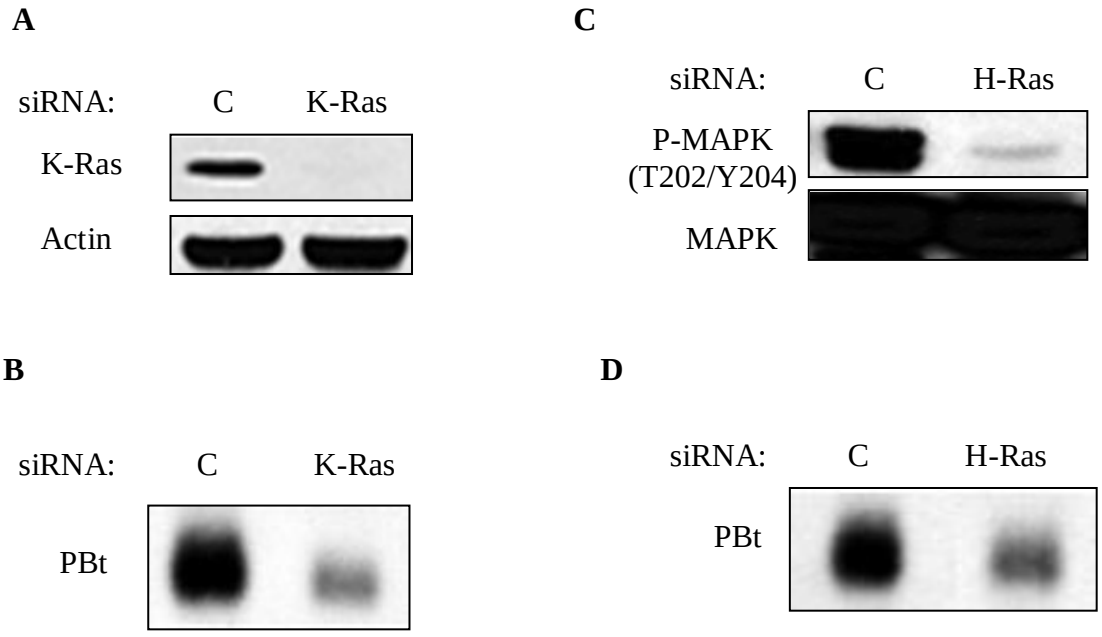

Figure 3.2.5-2: Knock-down of K-Ras or H-Ras reduces PLD activity. 30-50% confluent MDA-MB-231 cells were transfected with a nonspecific control siRNA, K-Ras or H-Ras siRNA and incubated for 48 hrs at 37°C and 5% CO_2. The media was then switched to DMEM supplemented with 0.5% serum and incubated overnight. Proteins were immunoblotted for (A) K-Ras and actin or (C) phosphorylated p44/p42 MAPK and total MAPK. (B, D) Lipids were collected and phosphatidylbutanol (PBt) production was determined.

3.2.6 PLD activity depends on RalA and Arf proteins

The small G-proteins RalA and Arf6 have been shown to be required for the induction of PLD activity in response to growth factors such as EGF [96], oncogenic Src [212] and Ras [210]. Although RalA interacts directly with PLD1, RalA is not sufficient to activate PLD1 [212, 285]. RalA and Arf6 actually worked synergistically to elevate PLD activity in H-Ras transformed cells [210]. Hence, our next objective was to determine if RalA and the Arf proteins, specifically Arf1 and Arf6, contribute to the elevated PLD activity observed in MDA-MB-231 cells.

We began by examining the effect of serum deprivation on active GTP-bound RalA. As shown in Fig. 3.2.6A, the levels of active RalA were unaffected when the cells were deprived of serum. Although this indicated that there was no correlation between GTP-bound RalA and PLD activity in 0.5% serum, there was still the possibility that RalA was required for PLD activity. To that end, we used the RalAS28N dominant negative mutant of RalA in which a serine (S) at codon 28 was replaced with an asparagine (N). Overexpression of RalAS28N reduced the PLD activity in 0.5% serum (Fig. 3.2.6B). Additionally, overexpression of dominant mutants of Arf1 (Arf1T31N) and Arf6 (ArfT27N) also reduced PLD activity (Fig. 3.2.6C). In these mutants a threonine (T) was replaced by an asparagine (N) at the indicated codon positions. Collectively, this data indicates that the induction of PLD as a result of serum withdrawal, depends on the activities of RalA, Arf1 and Arf6.

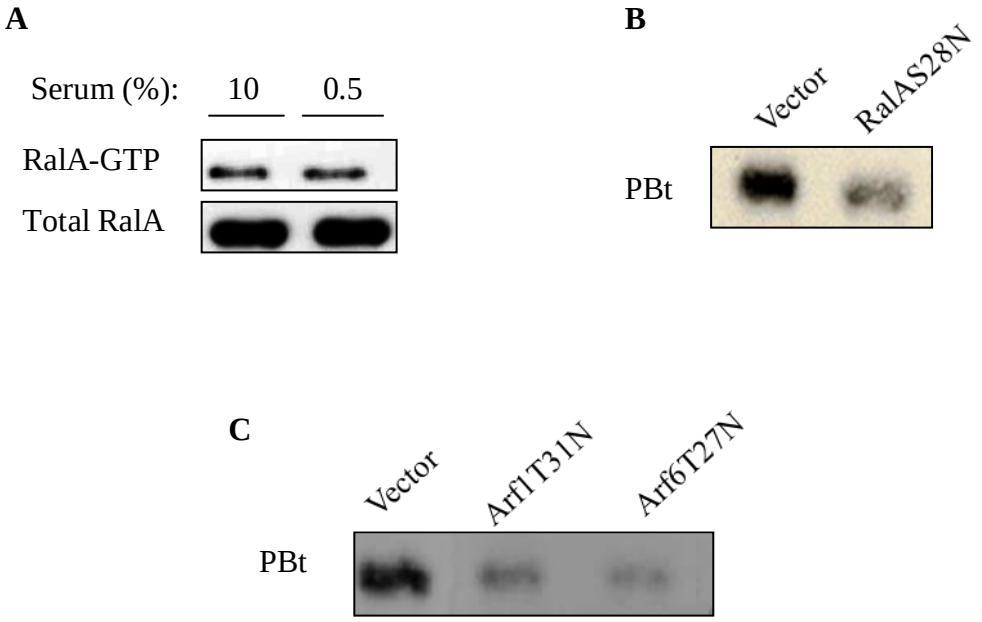

Figure 3.2.6: PLD activity depends on RalA, ARF1 and ARF6. (A) 90% confluent MDA-MB-231 cells was switched to DMEM supplemented with 10 % or 0.5% serum and incubated overnight. GTP- bound RalA was determined using a RalA activation assay kit. (B,C) 80% confluent MDA-MB-231 cells were transfected with an empty vector or a vector harboring (B) RalAS28N, (C) Arf1T31N or Arf6T27N and incubated overnight. The media was then switched to DMEM supplemented with 0.5% serum and after an additional overnight incubation, phosphatidylbutanol (PBt) production was determined.

3.3 Discussion

In this section, we provide evidence to show that the induction of PLD activity by low serum conditions depends on EGFR and the small G-proteins Ras, RalA, Arf1 and Arf6. In response to serum withdrawal, the MDA-MB-231 cells increase the activity and expression of EGFR (Fig. 3.2.2A). This induction of EGFR activity and expression contributes to the elevated PLD activity observed in these cells since suppression of EGFR function reduces PLD activity (Fig. 3.2.3-1C and Fig. 3.2.3-2B). These cells may be exhibiting one of the first "hallmarks of cancer" which is self sufficiency in growth signals. This property greatly reduces a tumor cell's dependence on exogenous growth stimulation [131]. One of the strategies used by tumor cells to achieve such autonomy involves altering the expression of growth factor receptors [131], a tactic that is clearly being exhibited by the MDA-MB-231 cells in low serum. The presence of such a minute amount of serum may be sufficient to activate EGFR signals as long as the receptor levels are elevated. This possibility was described by Fedi et al. who reported that receptor overexpression could render cancer cells hyperresponsive to generally insufficient growth factor levels [131, 292].

Since signals emanating from growth factor receptors are transmitted to molecules such as the GTPase Ras, we examined the GTP-bound status of this small protein. Our results show that like EGFR, the activation of Ras is also induced in serum deprived conditions (Fig. 3.2.4). Additionally, the elevation of GTP-loaded Ras was observed within 1 and 4 hours of serum deprivation–time points at which elevated PLD activity was also observed [130]. In a publication by Medema and Bos [293], they point out that in some tumors, mutant Ras proteins are able to unleash a stream of mitogenic signals

without continuous stimulation by their normal upstream regulators. PLD activity was also dependent on Ras signaling because inhibition of Ras activity reduced phosphatidylbutanol production (Fig. 3.2.5-1). Inhibition of either the activated mutant K-Ras or the wild-type H-Ras caused a reduction in PLD activity, suggesting that both the wild-type and mutant Ras proteins stimulate PLD activity (Fig. 3.2.5-2).

Another potential contributor to PLD activity in this system is the G-protein RalA, which is situated downstream of Ras but upstream of PLD. Assessment of the RalA-GTP status revealed that this G-protein does not respond to serum deprivation by elevating its activity (Fig. 3.2.6A). Although the RalA-GTP level remains unchanged, it does not necessarily mean that RalA is not playing a role in the induction of PLD activity. In fact, our lab has reported that active RalA alone is not sufficient to elevate PLD1 [285]. Furthermore, active RalA was demonstrated to work synergistically with the G-proteins Arf1 [294] and Arf6 [210] to elevate PLD activity. Transfection of dominant negative mutants of RalA, Arf1 and Arf6 into the MDA-MB-231 cells results in a decrease in PLD activity (Fig. 3.2.6). Altogether, we demonstrate that inhibition of the activities of RalA, Arf1 and Arf6 abrogate the PLD activity in the MDA-MB-231 cells.

CHAPTER IV

HONOKIOL PROMOTES APOPTOSIS VIA INHIBITION OF RAS AND PLD ACTIVITIES.

4.1 Introduction

Honokiol (Fig. 4.2.1A, [295]) is a naturally existing biphenyl compound which can be extracted from the root, stem, bark or seed cone of several Magnolia species such as *Magnolia grandiflora* [296] and *Magnolia officinalis* [297]. Traditional Chinese and Japanese herbal medicines have long used the bark of Magnolia trees for its antibacterial, antithrombosis and anxiolytic effects [296, 298, 299]. Its antitumor activities however, have only recently been described. For instance, honokiol exhibited strong cytotoxic activity against skin tumors, colon and lung cancers [300-302]. This potent biphenyl compound not only inhibited angiogenesis but it also caused a 50% reduction in tumor growth in a mouse model [303]. Treatment with honokiol has also been reported to induce apoptosis in B-cell chronic lymphocytic leukemia, multiple myeloma and breast cancer cells [304-306]. Finally, the multidrug resistant (MDR) breast cancer cell line MCF7/MDR, has been shown to be sensitive to honokiol [307].

Of significant interest is research done by Wolf et al. [306] who demonstrated that treatment of nude mice with honokiol completely arrested the growth of MDA-MB-231 xenografts. As a result of its potent antineoplastic properties, we sought to determine whether honokiol exerted its effects by targeting Ras and/or PLD activities. In this section we show that honokiol reduces Ras and PLD activities and induces apoptosis in cancer cells deprived of serum.

4.2 Results

4.2.1 Honokiol suppresses PLD activity

In addition to MDA-MB-231, the other cancer cell lines that respond to serum withdrawal by elevating their PLD activity are Calu-1 (lung) and T24 (bladder). Similar to the MDA-MB-231 cells, Calu-1 has an activating mutation in K-*ras* while the T24 cell line harbors an activating mutation in H-*ras*. Treatment of the Calu-1 cell line with honokiol resulted in a complete abrogation of PLD activity (Fig. 4.2.1B). This effect was also observed when the MDA-MB-231 and T24 cells were treated with honokiol (Fig. 4.2.1C). These results clearly show that honokiol functions as an effective inhibitor of PLD activity.

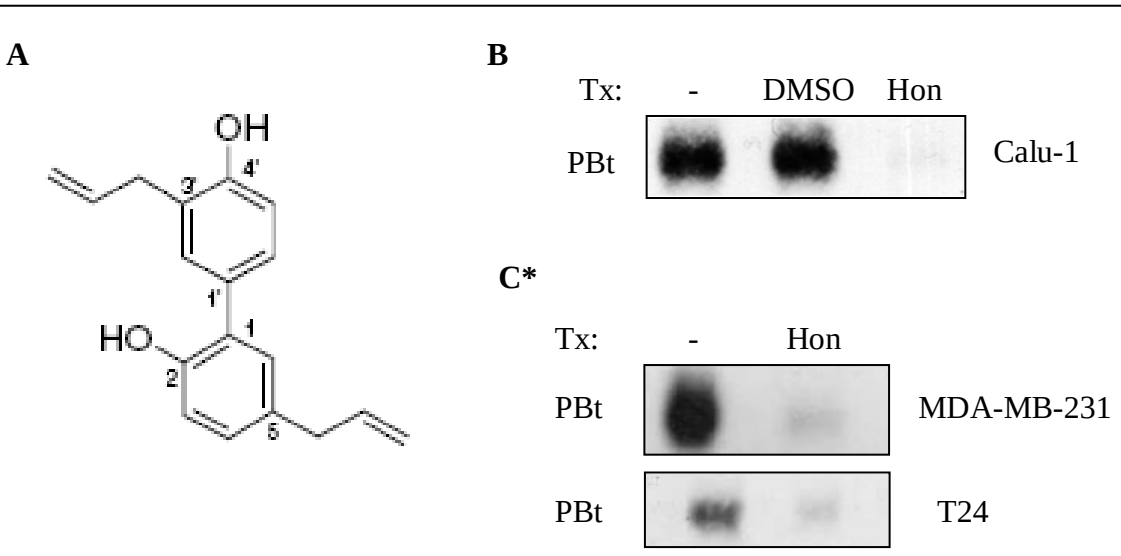

Figure 4.2.1: Effect of Honokiol on PLD activity in various cell lines. (A) Structure of honokiol (2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol). (B,C) 90% confluent cells (Calu-1, MDA-MB-231, and T24) were switched to media containing 0.5% serum and incubated overnight. Cells were treated with DMSO or 20 µM honokiol (Hon) where indicated for 4 hrs after which lipids were collected and phosphatidylbutanol(PBt) production was determined.(*: Y. Zheng)

4.2.2 Honokiol suppresses Ras activity

As demonstrated previously, the Ras activity of the MDA-MB-231 cells contributes to the elevated PLD activity observed in 0.5% serum. Therefore, compounds that suppress PLD activity may be doing so through Ras. To determine whether the effect of honokiol on PLD activity was mediated by Ras, we examined the effect of honokiol on Ras activity. Figure 4.2.2A shows that honokiol reduces the levels of GTP-bound Ras in 0.5% serum. In addition, Ras activity that was stimulated by EGF was also abrogated by honokiol (Fig. 4.2.2B). These results indicate that honokiol exerts its effects via inhibition of Ras.

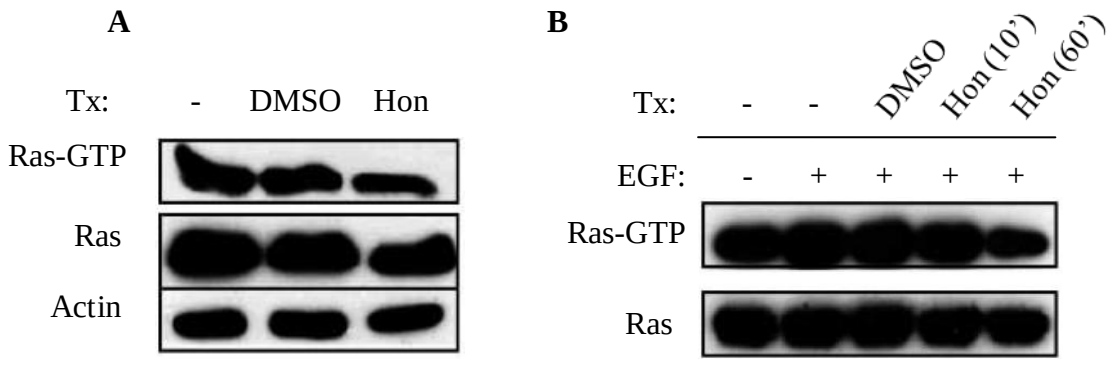

Figure 4.2.2: Honokiol suppresses Ras activity. 90% confluent MDA-MB-231 cells were switched to media containing 0.5% serum and incubated overnight. (A) Cells were then treated with DMSO or Honokiol for 2 hrs after which proteins lysates were used to pull down GTP-bound Ras and immunoblotted for Ras and actin. (B) Cells were pre-treated with DMSO (60 min) or 20 μM honokiol for the indicated times and EGF (200 ng/ml) was added 10 min before lysates were collected and the Ras assay performed.

4.2.3. Honokiol induces apoptosis in cancer cells deprived of serum

We have reported that the ability of MDA-MB-231 cells to induce their PLD activity under serum deprived conditions is essential for their survival. Since we demonstrated that honokiol suppressed both Ras and PLD activities, we examined its effect on survival. We show here that treatment with honokiol also induces apoptosis as shown by an increase in the cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 4.2.3).

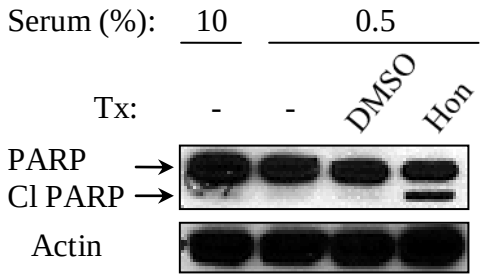

Figure 4.2.3: Honokiol induces apoptosis. 90% confluent MDA-MB-231 cells were switched to media containing 10% or 0.5% serum and incubated overnight. Cells were then treated with DMSO or 20 μM honokiol for 4 hrs after which lysates were immunoblotted for PARP (total and cleaved) and actin.

4.3 Discussion

Cancer cells with elevated PLD activity are less sensitive to the stress of serum withdrawal and are able to evade apoptosis. Once PLD activity is inhibited however, these cells lose their ability to cope and survive. Hence, targeting PLD may prove to be critical for the eradication of cancers that depend on PLD signaling for survival. The naturally occurring compound honokiol is emerging as a potent anticancer agent. Its effects have been attributed to its inhibition of angiogenic factors such as the vascular endothelial growth factor receptor R2 (KDR) [303] and survival pathways regulated by MAPK and PI3K.

In this chapter, we report that honokiol functions as a potent inhibitor of PLD activity in cell lines with stress-induced PLD activity. Additionally, honokiol suppressed the Ras activity in serum depleted conditions or the Ras activity induced by EGF stimulation. Cleavage of PARP was also induced when cells were treated with honokiol.

CHAPTER V

5`AMP-ACTIVATED PROTEIN KINASE MEDIATES THE INDUCTION OF PLD ACTIVITY AND MIGRATION UNDER SERUM-DEPRIVED CONDITIONS

5.1 Introduction

In our search to uncover additional proteins and pathways that possibly play a role in the induction of PLD activity under serum deprived conditions, we focused our attention on 5`AMP-activated protein kinase (AMPK). AMPK is a serine-threonine kinase that acts as a 'metabolic sensor' that responds to changes in cellular ATP levels [308]. Stressful conditions such as oxygen deprivation and heat shock, cause an increase in the AMP:ATP ratio which results in the activation of AMPK [235, 236]. Once activated, AMPK functions by inhibiting energy consuming processes while stimulating ATP-producing pathways. This way, AMPK works to optimize total cellular ATP levels in order to maintain critical physiological processes or for survival in response to extreme stress [309].

In our studies, the cancer cell lines are subjected to the stress of serum withdrawal and respond by elevating their PLD activity. Perusal of the literature revealed several reports that provide a link between PLD and AMPK activities. For instance, Hahn-Windgassen et al. [310] showed that depriving mouse embryo fibroblasts (MEFs) of serum (0.1% serum) caused an increase in the cellular AMP:ATP ratio and an increase in AMK activity. Significantly, treatment of L6 myotubes with the AMPK activator AICAR, was sufficient to elevate the PLD activity of this cell line [311].

Further examination of the literature revealed conflicting ideas on the function of AMPK as it relates to cell survival. Some reports indicate that AMPK has pro-apoptotic effects while others provide data that support the idea of an anti-apoptotic role for AMPK. Some researchers have observed that elevating AMPK activity leads to apoptosis

in gastric, lung and osteosarcoma [279, 280]. Conversely, other reports show that high AMPK activity protected both normal and tumoregenic cells from apoptosis triggered by nutrient or growth factor deprivation [282, 283].

Our investigations into the survival signals of cancer cells under serum deprived conditions reveal a role for AMPK in the regulation of PLD. In this section, we provide evidence to show that stimulation or suppression of AMPK results in the activation or inhibition of PLD activity respectively. Furthermore, inhibition of AMPK suppresses migration and induces apoptosis in low serum conditions.

5.2 Results

5.2.1 Correlation between AMPK and PLD activities in low serum

As mentioned previously, it has been demonstrated that depriving cells of growth factors and/or nutrients caused an increase in AMPK activity. To determine if the cell lines with elevated PLD activity respond in a similar manner, we subjected the MDA-MB-231 and Calu-1 cell lines to low serum conditions and examined its effect on AMPK activity. When activated, AMPK becomes phosphorylated on threonine 172 (Thr172), a property we used as an indicator of AMPK activity. As shown in figure 5.2.1-1A and B, the MDA-MB-231 as well as the Calu-1 cells respond to serum withdrawal by elevating their AMPK activity as demonstrated by the increase in P-AMPK (T172). Therefore, theses cells respond to serum deprivation by inducing both AMPK and PLD activities.

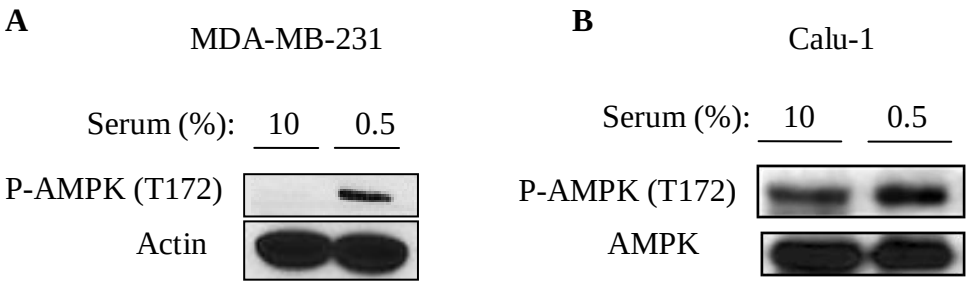

Figure 5.2.1-1: Effect of serum withdrawal on AMPK activity. 90% confluent MDA-MB-231 and Calu-1 cells were switched to media containing 10% or 0.5% serum and incubated overnight. (A) MDA-MB-231 lysates were immunoblotted for phosphorylated AMPK at threonine 172 (P-AMPK(T172) and actin while the lysates from (B) Calu-1 cells were immunoblotted for P-AMPK(T172) and AMPK.

Activation of AMPK results in an increase in GLUT4 protein levels [265]. As our results show, depriving cells of serum caused an increase in the expression levels of

GLUT4 (Fig. 5.2.1-2A). Some reports also demonstrate an inverse relationship between AMPK and mTOR activities [310]. mTOR exists as two complexes, mTORC1 and mTORC2. mTORC1 is believed to phosphorylate p70S6 kinase (S6K) while mTORC2 is thought to phosphorylate AKT [267]. To determine whether the inverse relationship was retained in our conditions, we checked the effect of serum withdrawal on mTOR activity in the MDA-MB-231 cell line. Indeed, serum deprivation caused a decrease in the activity of mTORC1 and mTORC2 as demonstrated by the lower levels of phosphorylated S6K and AKT in 0.5% serum (Fig. 5.2.1-2B). This indicates that the elevated AMPK activity observed in serum deprived conditions is accompanied by a decrease in mTOR activity despite the presence of high PLD activity.

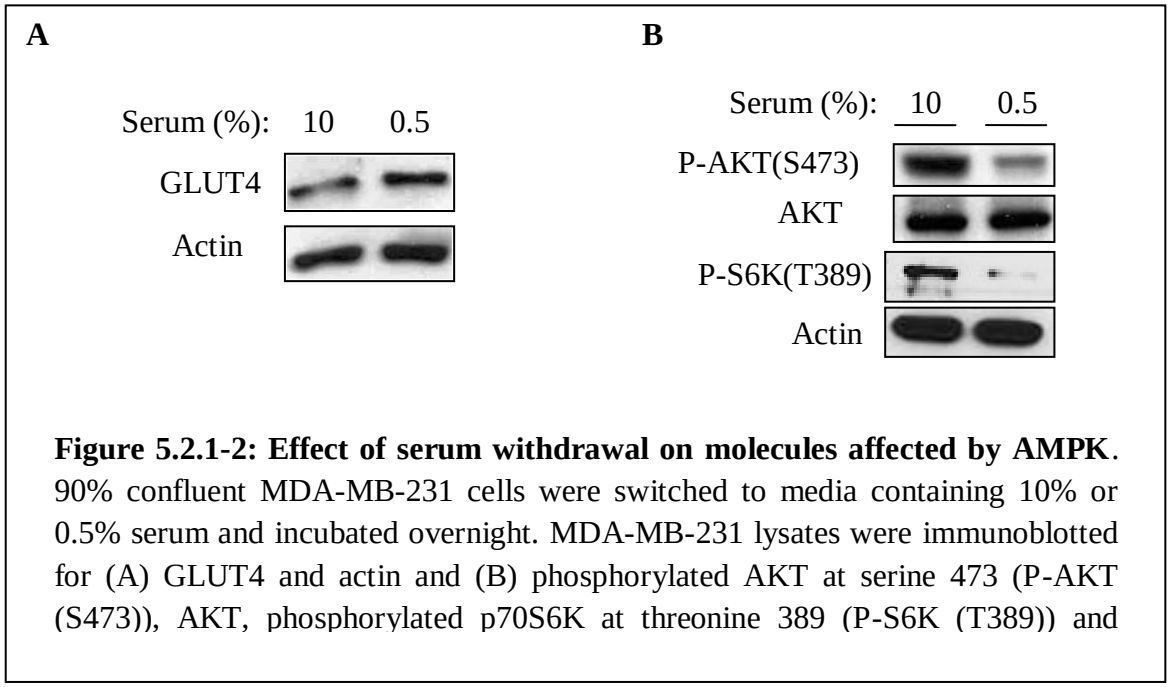

Figure 5.2.1-2: Effect of serum withdrawal on molecules affected by AMPK. 90% confluent MDA-MB-231 cells were switched to media containing 10% or 0.5% serum and incubated overnight. MDA-MB-231 lysates were immunoblotted for (A) GLUT4 and actin and (B) phosphorylated AKT at serine 473 (P-AKT (S473)), AKT, phosphorylated p70S6K at threonine 389 (P-S6K (T389)) and

A large amount of our knowledge about the effects of AMPK on cellular processes has been obtained through the use of 5-aminoimidazole 4-carboxamide riboside (AICAR), an activator of AMPK. When taken up by cells, AICAR is phosphorylated by

adenosine kinase to produce the monophosphorylated derivative ZMP which functions as an AMP mimetic [261] (Fig. 5.2.1-3A). We hypothesized that if the elevation in PLD activity in response to serum withdrawal is due to an increase in the AMP:ATP ratio, then mimicking an increase in AMP under high serum conditions would also stimulate PLD activity. To that end, we treated Calu-1 cells with AICAR and evaluated its effect on PLD activity. As hypothesized, introducing AICAR into cells in 10% serum resulted in a robust increase in PLD activity when compared to the untreated cells in 10% serum (Fig. 5.2.1-3B). The AICAR stimulated PLD activity however, was not as high as that observed in cells maintained in 0.5% serum. Taken together, these results indicate a correlation between the activities of AMPK and PLD.

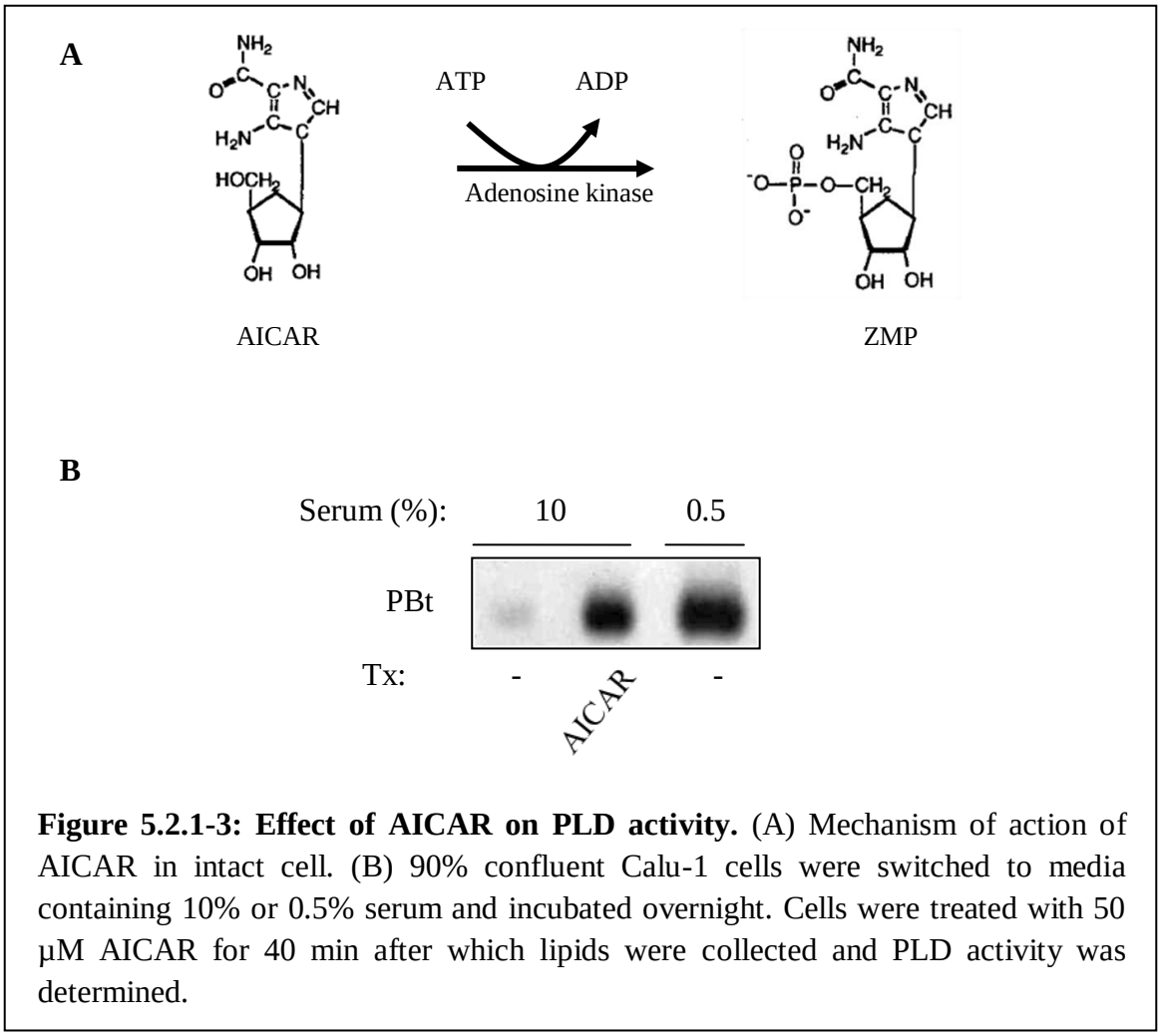

Figure 5.2.1-3: Effect of AICAR on PLD activity. (A) Mechanism of action of AICAR in intact cell. (B) 90% confluent Calu-1 cells were switched to media containing 10% or 0.5% serum and incubated overnight. Cells were treated with 50 µM AICAR for 40 min after which lipids were collected and PLD activity was determined.

5.2.2 PLD activity depends on AMPK

Since AMPK activity and signaling are activated under serum deprived conditions and mimicking an elevation in AMP levels stimulated PLD activity, we sought to determine if AMPK signaling is required for the induction of PLD activity in low serum. We initially approached this issue by using a pharmacological inhibitor of AMPK, 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine, commonly referred to as Compound C (CC) (Fig. 5.2.2-1A). This potent, selective and reversible inhibitor functions as an ATP competitor. As shown in figure 5.2.2-1B, CC

reduces the amount of active AMPK as demonstrated by the decreased levels of phosphorylated AMPK in the Calu-1 cells. Examination of the effect of CC on PLD activity revealed that this drug suppressed PLD activity in both Calu-1 and T24 cells (Fig. 5.2.2-1C and D).

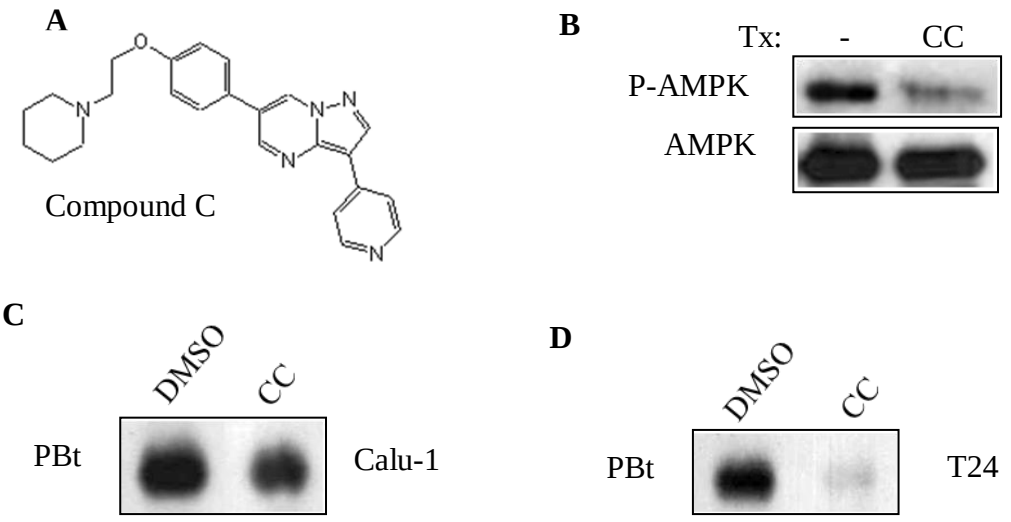

Figure 5.2.2-1: Pharmacological inhibition of AMPK reduces PLD activity. (A) Structure of Compound C. (B-D) 90% confluent cells were switched to media containing 0.5% serum at which point DMSO or 20 µM Compound C (CC) was added and the cells were incubated overnight. (B) Calu-1 lysates were immunoblotted for phosphorylated AMPK (T172) and total AMPK. (C and D) Lipids were collected from Calu-1 and T24 cells and PLD activity was

To further assess the requirement for AMPK in stress-induced PLD activity, we utilized siRNA to knock down AMPK and LKB1. Phosphorylation of AMPK at threonine 172 is critical for its enzymatic activity and LKB1 has been identified as the primary kinase responsible for this phosphorylation on AMPK [251, 252]. We hypothesized that a reduction in AMPK and LKB1 protein levels, which will effectively reduce AMPK activity, will also result in a reduction of PLD activity. Certainly, as figure 5.2.2-2 A and B show, knock-down of LKB1 caused a decrease in PLD activity and a

more pronounced effect was observed when AMPK was knocked out. These results demonstrate the dependence of PLD activity on signaling from AMPK.

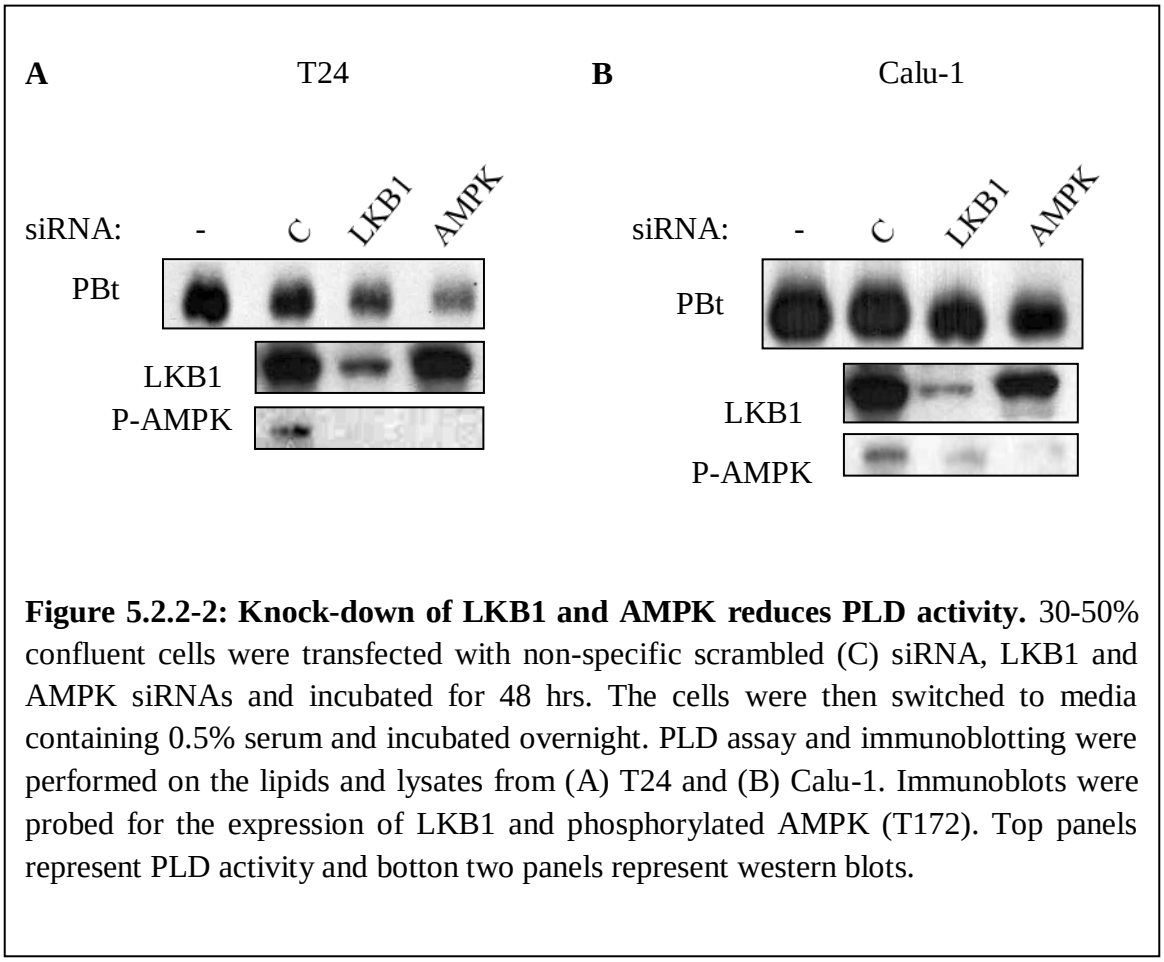

Figure 5.2.2-2: Knock-down of LKB1 and AMPK reduces PLD activity. 30-50% confluent cells were transfected with non-specific scrambled (C) siRNA, LKB1 and AMPK siRNAs and incubated for 48 hrs. The cells were then switched to media containing 0.5% serum and incubated overnight. PLD assay and immunoblotting were performed on the lipids and lysates from (A) T24 and (B) Calu-1. Immunoblots were probed for the expression of LKB1 and phosphorylated AMPK (T172). Top panels represent PLD activity and botton two panels represent western blots.

5.2.3 AMPK does not signal through Ras

As shown Chapter III, the PLD activity induced by serum depleted conditions was mediated by EGFR and Ras signaling. Hence, we sought to determine whether the effects of AMPK on PLD were mediated by EGFR and Ras signaling or vice versa. To start, we treated MDA-MB-231 cells with Compound C and AG1478, chemicals that inhibit AMPK and EGFR respectively. If EGFR and Ras signaling are positioned upstream of AMPK, then treatment with AG1478 would suppress AMPK activity. The results

however, show that while AMPK activity is reduced by CC, the phosphorylated levels of AMPK were unaffected by AG1478 (Fig. 5.2.3-1).

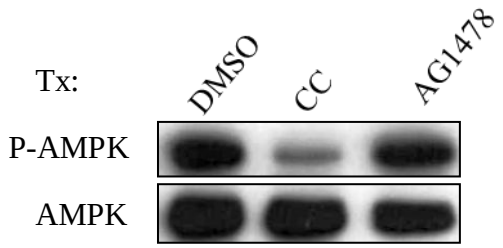

Figure 5.2.3-1: AMPK is not responsive to AG1478. 90% confluent MDA-MB-231 cells were switched to media containing 0.5% serum at which point DMSO or 20 µM Compound C was added and the cells incubated overnight. Forty minutes before lysates were collected, 250 nM AG1478 was added. Lysates were immunoblotted and probed for phosphorylated AMPK (T172) and AMPK.

Inspection of the effects of AICAR and CC on Ras activity revealed similar results. To determine if AMPK activated PLD in a Ras dependent manner, we assayed the effect of Compound C and AICAR on Ras activity. The results show that inhibition of AMPK with CC (Fig. 5.2.3-2A) or stimulation of AMPK with AICAR (Fig. 5.2.3-2B) had no effect on Ras activity. These results indicate that the effects of AMPK and EGFR/Ras on PLD activity were occurring via distinct pathways.

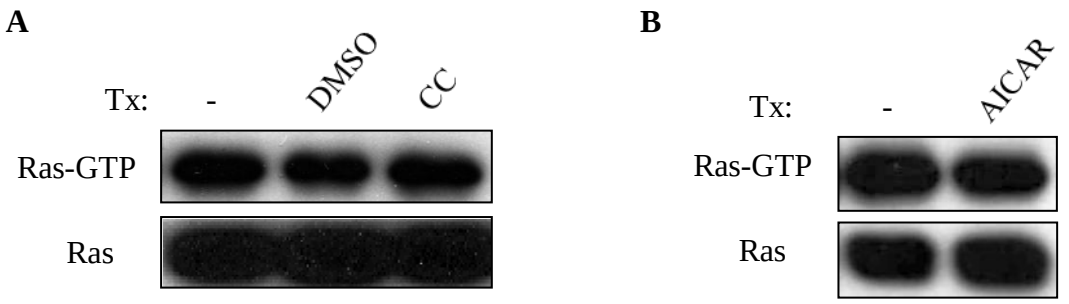

Figure 5.2.3-2: Effect of AICAR and Compound C on Ras activity. 90% confluent MDA-MB-231 cells were switched to media containing 0.5% serum at which point (A) DMSO and 20 µM Compound C (CC) were added and cells were incubated overnight. (B) Forty minutes before lysates were collected cells were treated with 50 µM AICAR. In both A and B, the amount of GTP bound and total Ras was determined.

5.2.4 Suppression of AMPK inhibits migration

Our lab has reported that subjecting several cell lines to the stress of serum withdrawal causes an increase in the ability of these cells to migrate and invade [130]. Additionally, the migration of these cell lines depended on PLD activity. To determine whether their migration is dependent on AMPK, we treated MDA-MB-231, T24 and Calu-1 cells lines with Compound C and examined its effects on cell migration. As shown in Figure 5.2.4, suppression of AMPK activity caused a dramatic decrease in migration in the three cell lines tested. Additionally, suppression of AMPK induced apoptosis which was demonstrated by the increase in PARP cleavage in cells treated with Compound C (data not shown).

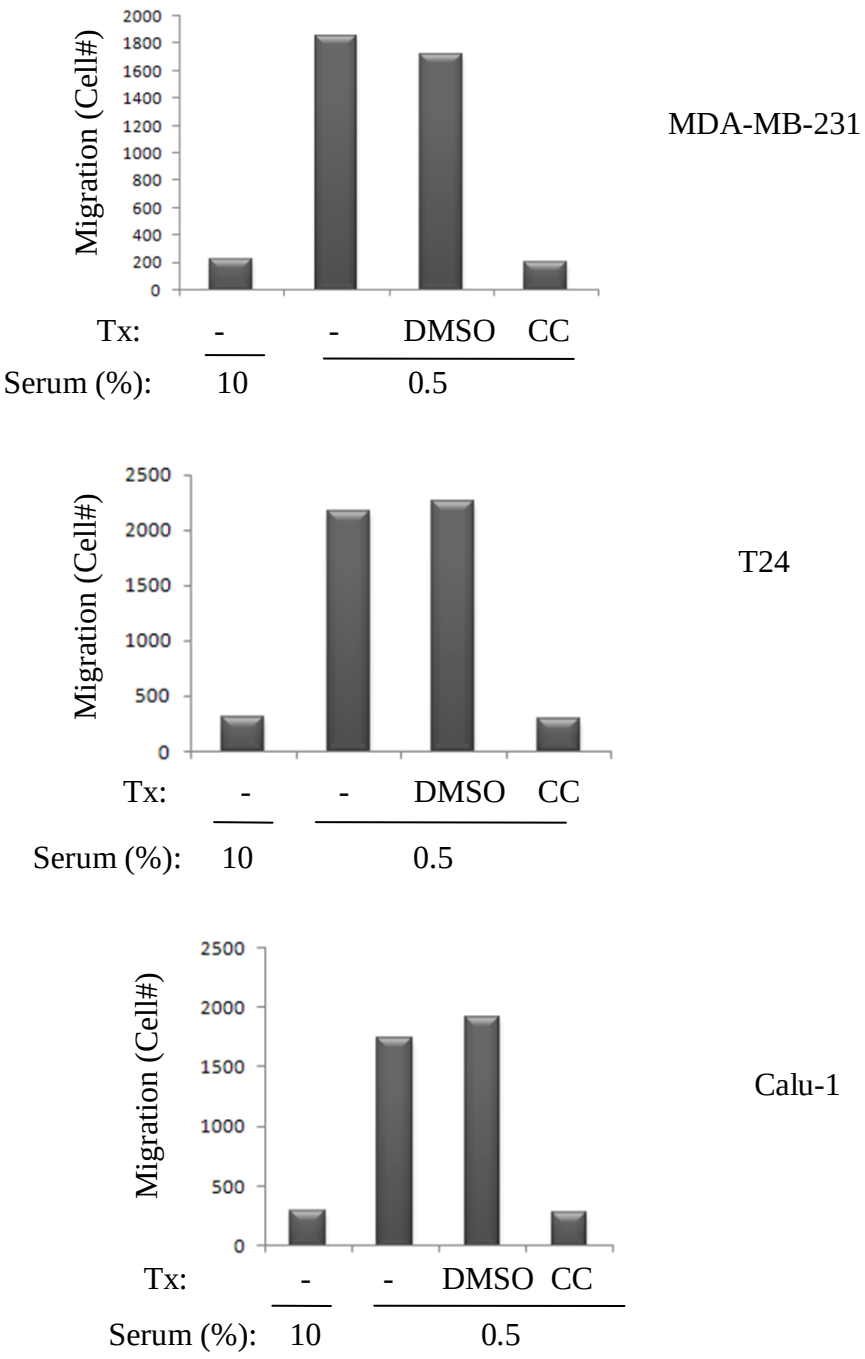

Figure 5.2.4: Suppression of AMPK inhibits migration. Suspensions of each cell line were placed in BioCoat^{TM} cell culture inserts in media containing 10% or 0.5% serum with or without DMSO or 20 µM Compound C (CC). 24 hrs later, cells that had migrated to the lower surface of the filter were counted as described in Materials and Methods 2.2.6. Each assay was performed three times with equivalent results.

5.3 Discussion

Cells within an emerging tumor are exposed to environments in which the nutrient, growth factor and oxygen supplies are insufficient to maintain proper homeostasis. That being said, the ability of some cells to tolerate this stressful environment may be a critical factor in contributing to tumor progression [283]. The elevation of AMPK activity has been implicated as a mechanism for survival under stressful conditions that result in an increase in the AMP:ATP ratio [283]. Since the induction of PLD activity is critical for the survival of certain cancer cells exposed to serum-limiting conditions, we sought to determine if AMPK played a role in PLD's response.

Our results show that depriving cells of serum causes an increase in AMPK and PLD activities (Fig. 5.2.1-1). When activated, AMPK inhibits energy consuming processes controlled by mTOR [312]. We have shown that the induction of AMPK activity is accompanied by a decrease in mTOR (both complex1 and complex 2) activity as seen by the reduction in the activation of AKT and S6K in low serum (Fig. 5.2.1-2). The paradox that this data creates involves already established connections between PLD and mTOR. For instance, phosphatidic acid (PA) was reported to be required for the activation of mTOR [100, 313]. PA was also shown to compete with rapamycin for binding to mTOR [313]. Additionally, our lab has shown that elevated PLD activity confers resistance to rapamycin [136]. Taken together, these published reports implicate mTOR as a downstream target of PLD, so why is it that stressful conditions that elevate PLD activity results in a decrease in mTOR activity? We surmise that phosphatidic acid may be necessary but not sufficient to activate mTOR.

Further examination of the relationship between AMPK and PLD revealed that in 10% serum, the addition of AICAR to mimic an increase in AMP levels caused an elevation in PLD activity which was significantly higher than that of the control cells (Fig. 5.2.1-3). This data indicates that PLD is sensitive to changes in a cell's energy levels. Conversely, pharmacological inhibition of AMPK activity resulted in a decrease in PLD activity (Fig. 5.2.2-1). An effect that was also observed when AMPK activity was suppressed by siRNA targeting LKB1 and AMPK (Fig. 5.2.2-2). These results indicate that PLD's response to serum deprivation is dependent on AMPK.

We had demonstrated previously that the serum-withdrawal induced PLD activity depended on signaling from the EGFR/Ras pathway. Hence we sought to determine whether the AMPK and EGFR/Ras signals were integrated. Inhibition of EGFR/Ras by the EGFR inhibitor AG1478 did not affect the activity of AMPK. Similarly, suppression of AMPK activity with Compound C did not alter the activation state of Ras. These results indicate that AMPK and EGFR/Ras signal to PLD through two distinct pathways.

Serum deprivation has been shown to induce migration in several of the cell lines, a property that was dependent on PLD activity [130]. AMPK is also required for this stress induced migration since inhibition of AMPK suppressed this response. Lastly, PLD turns on survival signals in serum deprived conditions as inhibition of PLD results in cell death. Likewise, AMPK is required for survival in serum-depleted conditions since inhibition of AMPK leads to apoptosis (data not shown). Collectively, the data suggests that AMPK acts in concert with PLD as part of an adaptive response to serum-withdrawal in order to help cells survive.

CHAPTER VI

Conclusion

The overall objective of this work was to investigate the molecules and pathways that play a role in the induction of PLD activity in response to serum withdrawal. Concomitant with an induction of PLD activity, we observed an increase in the activation of EGFR and Ras. Signals emanating from these molecules are important for PLD activity since suppression of EGFR or Ras results in a decrease in PLD activity. Inhibition of the activities of the small G proteins RalA, Arf1 and Arf6, which are situated downstream of Ras, also decreased PLD activity.

Interestingly, we have been able to induce PLD activity in 10% serum by exposing cancer cells to conditions that mimic an increase in AMP concentrations. This data indicated that proteins that respond to changes in the AMP:ATP ratio may be involved in regulating PLD activity. We have shown that like PLD, the activity of the energy sensing enzyme AMPK is also induced by serum withdrawal. This induction of AMPK activity is accompanied by a decrease in mTOR activity and an increase in GLUT4 expression. PLD activity depends on AMPK signaling since suppression of this serine-threonine kinase or its upstream kinase LKB1, abrogates PLD activity. In response to serum withdrawal, the cancer cell lines used in this study display an increase in migration-a property that was dependent on PLD activity [130]. Here, we have demonstrated that the migration of the MDA-MB-231, T24 and Calu-1 cell lines also depend on AMPK.

Because of its contribution to survival, migration and invasion, PLD has emerged as a promising target in the fight against cancer. In collaboration with laboratories at Emory University and NYU Medical Center, we have investigated the mechanism of action of the naturally existing compound Honokiol. As described in Chapter IV, this

potent compound exhibits antitumor activity *in vivo* and *in vitro*. Further examination of Honokiol's effects reveal that this compound suppresses Ras and PLD activities and induces apoptosis in the cancer cell lines tested [314].

CHAPTER VII

References

1. Hanahan, D.J. and I.L. Chaikoff, *THE PHOSPHORUS-CONTAINING LIPIDES OF THE CARROT*

in *J. Biol. Chem.* 1947. p. 233-240.
2. Hanahan, D.J. and I.L. Chaikoff, *ON THE NATURE OF THE PHOSPHORUS-CONTAINING LIPIDES OF CABBAGE LEAVES AND THEIR RELATION TO A PHOSPHOLIPIDE-SPLITTING ENZYME CONTAINED IN THESE LEAVES.* J. Biol. Chem., 1948. **172**(1): p. 191-198.
3. Saito, M. and J. Kanfer, *Phosphatidohydrolase activity in a solubilized preparation from rat brain particulate fraction.* Arch Biochem Biophys, 1975. **169**(1): p. 318-23.
4. Abousalham, A., et al., *Improved purification and biochemical characterization of phospholipase D from cabbage.* Biochim Biophys Acta, 1993. **1158**(1): p. 1-7.
5. Wang, X.M., J.H. Dyer, and L. Zheng, *Purification and Immunological Analysis of Phospholipase D from Castor Bean Endosperm.* Archives of Biochemistry and Biophysics, 1993. **306**(2): p. 486-494.
6. Wang, X., L. Xu, and L. Zheng, *Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from Ricinus communis L.* J. Biol. Chem., 1994. **269**(32): p. 20312-20317.
7. Ueki, J., et al., *Purification and characterization of phospholipase D (PLD) from rice (Oryza sativa L.) and cloning of cDNA for PLD from rice and maize (Zea mays L.).* Plant Cell Physiol, 1995. **36**(5): p. 903-14.
8. Qin, W., K. Pappan, and X. Wang, *Molecular heterogeneity of phospholipase D (PLD). Cloning of PLDgamma and regulation of plant PLDgamma, -beta, and -alpha by polyphosphoinositides and calcium.* J Biol Chem, 1997. **272**(45): p. 28267-73.
9. Cha, Y.R., et al., *Cloning of a cDNA encoding phospholipase D from Pimpinella brachycarpa.* Mol Cells, 1998. **8**(1): p. 19-26.
10. Rose, K., et al., *Phospholipase D signaling is essential for meiosis.* Proc Natl Acad Sci U S A, 1995. **92**(26): p. 12151-5.
11. Ella, K.M., J.W. Dolan, and K.E. Meier, *Characterization of a regulated form of phospholipase D in the yeast Saccharomyces cerevisiae.* Biochem J, 1995. **307 (Pt 3)**: p. 799-805.
12. Ella, K.M., et al., *Characterization of Saccharomyces cerevisiae deficient in expression of phospholipase D.* Biochem J, 1996. **314 (Pt 1)**: p. 15-9.
13. Waksman, M., et al., *Identification and characterization of a gene encoding phospholipase D activity in yeast.* J Biol Chem, 1996. **271**(5): p. 2361-4.
14. Brown, H.A., et al., *Partial purification and characterization of Arf-sensitive phospholipase D from porcine brain.* J Biol Chem, 1995. **270**(25): p. 14935-43.
15. Hammond, S.M., et al., *Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family.* J Biol Chem, 1995. **270**(50): p. 29640-3.
16. Katayama, K., et al., *Cloning, differential regulation and tissue distribution of alternatively spliced isoforms of ADP-ribosylation-factor-dependent phospholipase D from rat liver.* Biochem J, 1998. **329 (Pt 3)**: p. 647-52.

17. Kodaki, T. and S. Yamashita, *Cloning, expression, and characterization of a novel phospholipase D complementary DNA from rat brain.* J Biol Chem, 1997. **272**(17): p. 11408-13.
18. Hammond, S.M., et al., *Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha.* J Biol Chem, 1997. **272**(6): p. 3860-8.
19. Colley, W.C., et al., *Cloning and expression analysis of murine phospholipase D1.* Biochem J, 1997. **326 (Pt 3)**: p. 745-53.
20. Colley, W.C., et al., *Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization.* Curr Biol, 1997. **7**(3): p. 191-201.
21. McDermott, M., M.J. Wakelam, and A.J. Morris, *Phospholipase D.* Biochem Cell Biol, 2004. **82**(1): p. 225-53.
22. Sung, T.C., et al., *Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity.* EMBO J, 1997. **16**(15): p. 4519-30.
23. Sciorra, V.A., et al., *Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes.* J Cell Biol, 2002. **159**(6): p. 1039-49.
24. Sugars, J.M., et al., *Hierarchy of membrane-targeting signals of phospholipase D1 involving lipid modification of a pleckstrin homology domain.* J Biol Chem, 2002. **277**(32): p. 29152-61.
25. Sung, T.C., et al., *Structural analysis of human phospholipase D1.* J Biol Chem, 1999. **274**(6): p. 3659-66.
26. Sung, T.C., et al., *Molecular analysis of mammalian phospholipase D2.* J Biol Chem, 1999. **274**(1): p. 494-502.
27. Sciorra, V.A., et al., *Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes.* EMBO J, 1999. **18**(21): p. 5911-21.
28. Du, G., et al., *Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs.* J. Cell Biol., 2003. **162**(2): p. 305-315.
29. Xu, Y., et al., *The Phox homology (PX) domain, a new player in phosphoinositide signalling.* Biochem J, 2001. **360**(Pt 3): p. 513-30.
30. Freyberg, Z., et al., *Intracellular localization of phospholipase D1 in mammalian cells.* Mol Biol Cell, 2001. **12**(4): p. 943-55.
31. Toda, K., et al., *Colocalization of phospholipase D1 and GTP-binding-defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes.* FEBS Lett, 1999. **442**(2-3): p. 221-5.
32. Hughes, W.E. and P.J. Parker, *Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity.* Biochem J, 2001. **356**(Pt 3): p. 727-36.
33. Huang, P., et al., *Insulin-stimulated plasma membrane fusion of Glut4 glucose transporter-containing vesicles is regulated by phospholipase D1.* Mol Biol Cell, 2005. **16**(6): p. 2614-23.

34. Brown, F.D., et al., *Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation.* Curr Biol, 1998. **8**(14): p. 835-8.
35. Du, G., et al., *Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis.* Mol Biol Cell, 2004. **15**(3): p. 1024-30.
36. Honda, A., et al., *Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation.* Cell, 1999. **99**(5): p. 521-32.
37. Divecha, N., et al., *Interaction of the type Ialpha PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4, 5-bisphosphate in the regulation of PLD2 activity.* EMBO J, 2000. **19**(20): p. 5440-9.
38. Lee, S., et al., *Actin directly interacts with phospholipase D, inhibiting its activity.* J Biol Chem, 2001. **276**(30): p. 28252-60.
39. Exton, J.H., *Phospholipase D: enzymology, mechanisms of regulation, and function.* Physiol Rev, 1997. **77**(2): p. 303-20.
40. Kim, J.H., et al., *Cytosolic phospholipase A2-mediated regulation of phospholipase D2 in leukocyte cell lines.* J Immunol, 1999. **163**(10): p. 5462-70.
41. Billah, M.M., et al., *Regulation of phospholipase D in HL-60 granulocytes. Activation by phorbol esters, diglyceride, and calcium ionophore via protein kinase- independent mechanisms.* J Biol Chem, 1989. **264**(15): p. 9069-76.
42. Meats, J.E., L. Steele, and J.G. Bowen, *Identification of phospholipase D (PLD) activity in mouse peritoneal macrophages.* Agents Actions, 1993. **39 Spec No**: p. C14-6.
43. Ueno, N., M. Murakami, and I. Kudo, *Functional crosstalk between phospholipase D(2) and signaling phospholipase A(2)/cyclooxygenase-2-mediated prostaglandin biosynthetic pathways.* FEBS Lett, 2000. **475**(3): p. 242-6.
44. Martinson, E.A., et al., *Platelet phospholipase D is activated by protein kinase C via an integrin alpha IIb beta 3-independent mechanism.* Biochem J, 1995. **310 (Pt 2)**: p. 623-8.
45. Kessels, G.C., D. Roos, and A.J. Verhoeven, *fMet-Leu-Phe-induced activation of phospholipase D in human neutrophils. Dependence on changes in cytosolic free Ca2+ concentration and relation with respiratory burst activation.* J Biol Chem, 1991. **266**(34): p. 23152-6.
46. Denys, A., et al., *Thapsigargin-stimulated MAP kinase phosphorylation via CRAC channels and PLD activation: inhibitory action of docosahexaenoic acid.* FEBS Lett, 2004. **564**(1-2): p. 177-82.
47. Brizuela, L., et al., *Sphingosine 1-phosphate: a novel stimulator of aldosterone secretion.* J Lipid Res, 2006. **47**(6): p. 1238-49.
48. Exton, J.H., *Phospholipase D.* Biochim Biophys Acta, 1998. **1436**(1-2): p. 105-15.
49. Min, D.S., S.K. Park, and J.H. Exton, *Characterization of a rat brain phospholipase D isozyme.* J Biol Chem, 1998. **273**(12): p. 7044-51.
50. Bosch, R.R., et al., *Concerted action of cytosolic Ca2+ and protein kinase C in receptor-mediated phospholipase D activation in Chinese hamster ovary cells*

expressing the cholecystokinin-A receptor. Biochem J, 1999. **337 (Pt 2)**: p. 263-8.

51. Khan, N.A. and A. Hichami, *Ionotrophic 5-hydroxytryptamine type 3 receptor activates the protein kinase C-dependent phospholipase D pathway in human T-cells.* Biochem J, 1999. **344 Pt 1**: p. 199-204.
52. Slaaby, R., et al., *Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293 cells mediated by the phospholipase C gamma and protein kinase C alpha signalling cascade.* Biochem J, 2000. **351 Pt 3**: p. 613-9.
53. Pai, J.K., E.A. Dobek, and W.R. Bishop, *Endothelin-1 activates phospholipase D and thymidine incorporation in fibroblasts overexpressing protein kinase C beta 1.* Cell Regul, 1991. **2**(11): p. 897-903.
54. Pai, J.K., et al., *Overexpression of protein kinase C beta 1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts.* Proc Natl Acad Sci U S A, 1991. **88**(2): p. 598-602.
55. Pachter, J.A., et al., *Differential regulation of phosphoinositide and phosphatidylcholine hydrolysis by protein kinase C-beta 1 overexpression. Effects on stimulation by alpha-thrombin, guanosine 5'-O-(thiotriphosphate), and calcium.* J Biol Chem, 1992. **267**(14): p. 9826-30.
56. Eldar, H., et al., *Up-regulation of phospholipase D activity induced by overexpression of protein kinase C-alpha. Studies in intact Swiss/3T3 cells and in detergent-solubilized membranes in vitro.* J Biol Chem, 1993. **268**(17): p. 12560-4.
57. Han, J.M., et al., *Phosphorylation-dependent regulation of phospholipase D2 by protein kinase C delta in rat Pheochromocytoma PC12 cells.* J Biol Chem, 2002. **277**(10): p. 8290-7.
58. Hornia, A., et al., *Antagonistic effects of protein kinase C alpha and delta on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor.* Mol Cell Biol, 1999. **19**(11): p. 7672-80.
59. Brown, H.A., et al., *ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity.* Cell, 1993. **75**(6): p. 1137-44.
60. Liscovitch, M., et al., *Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase D.* J Biol Chem, 1994. **269**(34): p. 21403-6.
61. Lopez, I., R.S. Arnold, and J.D. Lambeth, *Cloning and initial characterization of a human phospholipase D2 (hPLD2). ADP-ribosylation factor regulates hPLD2.* J Biol Chem, 1998. **273**(21): p. 12846-52.
62. Hawkins, P.T., T.R. Jackson, and L.R. Stephens, *Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase.* Nature, 1992. **358**(6382): p. 157-9.
63. Kim, J.H., et al., *Selective activation of phospholipase D2 by unsaturated fatty acid.* FEBS Lett, 1999. **454**(1-2): p. 42-6.
64. Voss, M., et al., *Phospholipase D stimulation by receptor tyrosine kinases mediated by protein kinase C and a Ras/Ral signaling cascade.* J Biol Chem, 1999. **274**(49): p. 34691-8.

65. Cook, S.J. and M.J. Wakalam, *Epidermal growth factor increases sn-1,2-diacylglycerol levels and activates phospholipase D-catalysed phosphatidylcholine breakdown in Swiss 3T3 cells in the absence of inositol-lipid hydrolysis.* Biochemistry Journal, 1992. **285**: p. 247-253.
66. Kaszkin, M., et al., *Epidermal-growth-factor-induced production of phosphatidylalcohols by HeLa cells and A431 cells through activation of phospholipase D.* Biochem J, 1992. **287 (Pt 1)**: p. 51-7.
67. Song, J., Y.W. Jiang, and D.A. Foster, *Epidermal growth factor induces the production of biologically distinguishable diglyceride species from phosphatidylinositol and phosphatidylcholine via the independent activation of type C and type D phospholipases.* Cell Growth Differ, 1994. **5**(1): p. 79-85.
68. Shen, Y., L. Xu, and D.A. Foster, *Role for phospholipase D in receptor-mediated endocytosis.* Mol Cell Biol, 2001. **21**(2): p. 595-602.
69. Hess, J.A., et al., *Role of Rho family proteins in phospholipase D activation by growth factors.* J Biol Chem, 1997. **272**(3): p. 1615-20.
70. Yeo, E.J. and J.H. Exton, *Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells.* J Biol Chem, 1995. **270**(8): p. 3980-8.
71. Ahmed, A., et al., *Basic FGF activates phospholipase D in endothelial cells in the absence of inositol-lipid hydrolysis.* Am J Physiol, 1994. **266**(1 Pt 1): p. C206-12.
72. Cross, M.J., et al., *Tyrosine 766 in the fibroblast growth factor receptor-1 is required for FGF-stimulation of phospholipase C, phospholipase D, phospholipase A(2), phosphoinositide 3-kinase and cytoskeletal reorganisation in porcine aortic endothelial cells.* J Cell Sci, 2000. **113 (Pt 4)**: p. 643-51.
73. Cho, C.H., et al., *Localization of VEGFR-2 and PLD2 in endothelial caveolae is involved in VEGF-induced phosphorylation of MEK and ERK.* Am J Physiol Heart Circ Physiol, 2004. **286**(5): p. H1881-8.
74. Donchenko, V., A. Zannetti, and P.M. Baldini, *Insulin-stimulated hydrolysis of phosphatidylcholine by phospholipase C and phospholipase D in cultured rat hepatocytes.* Biochim Biophys Acta, 1994. **1222**(3): p. 492-500.
75. Standaert, M.L., et al., *Insulin stimulates phospholipase D-dependent phosphatidylcholine hydrolysis, Rho translocation, de novo phospholipid synthesis, and diacylglycerol/protein kinase C signaling in L6 myotubes.* Endocrinology, 1996. **137**(7): p. 3014-20.
76. Li, H.S., et al., *The guanine nucleotide exchange factor ARNO mediates the activation of ARF and phospholipase D by insulin.* BMC Cell Biol, 2003. **4**: p. 13.
77. Shome, K., C. Vasudevan, and G. Romero, *ARF proteins mediate insulin-dependent activation of phospholipase D.* Curr Biol, 1997. **7**(6): p. 387-96.
78. Slaaby, R., et al., *PLD2 complexes with the EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist stimulation.* J Biol Chem, 1998. **273**(50): p. 33722-7.
79. Min, D.S., E.G. Kim, and J.H. Exton, *Involvement of tyrosine phosphorylation and protein kinase C in the activation of phospholipase D by H2O2 in Swiss 3T3 fibroblasts.* J Biol Chem, 1998. **273**(45): p. 29986-94.

80. Rydzewska, G. and J. Morisset, *Activation of pancreatic acinar cell phospholipase D by epidermal, insulin-like, and basic fibroblast growth factors involves tyrosine kinase.* Pancreas, 1995. **10**(1): p. 59-65.
81. Natarajan, V., W.M. Scribner, and S. Vepa, *Regulation of phospholipase D by tyrosine kinases.* Chem Phys Lipids, 1996. **80**(1-2): p. 103-16.
82. Natarajan, V., W.M. Scribner, and S. Vepa, *Phosphatase inhibitors potentiate 4-hydroxynonenal-induced phospholipase D activation in vascular endothelial cells.* Am J Respir Cell Mol Biol, 1997. **17**(2): p. 251-9.
83. Anthes, J.C., et al., *Granulocyte phospholipase D is activated by a guanine nucleotide dependent protein factor.* Biochem Biophys Res Commun, 1991. **175**(1): p. 236-43.
84. Olson, S.C., E.P. Bowman, and J.D. Lambeth, *Phospholipase D activation in a cell-free system from human neutrophils by phorbol 12-myristate 13-acetate and guanosine 5'-O-(3-thiotriphosphate). Activation is calcium dependent and requires protein factors in both the plasma membrane and cytosol.* J Biol Chem, 1991. **266**(26): p. 17236-42.
85. Geny, B. and S. Cockcroft, *Synergistic activation of phospholipase D by protein kinase C- and G-protein-mediated pathways in streptolysin O-permeabilized HL60 cells.* Biochem J, 1992. **284 (Pt 2)**: p. 531-8.
86. Geny, B., A. Fensome, and S. Cockcroft, *Rat brain cytosol contains a factor which reconstitutes guanine-nucleotide-binding-protein-regulated phospholipase-D activation in HL60 cells previously permeabilized with streptolysin O.* Eur J Biochem, 1993. **215**(2): p. 389-96.
87. Dascher, C. and W.E. Balch, *Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus.* J Biol Chem, 1994. **269**(2): p. 1437-48.
88. Chung, J.K., et al., *Synaptojanin inhibition of phospholipase D activity by hydrolysis of phosphatidylinositol 4,5-bisphosphate.* J Biol Chem, 1997. **272**(25): p. 15980-5.
89. Lukowski, S., et al., *Inhibition of phospholipase D activity by fodrin. An active role for the cytoskeleton.* J Biol Chem, 1996. **271**(39): p. 24164-71.
90. Lee, C., et al., *Inhibition of phospholipase D by clathrin assembly protein 3 (AP3).* J Biol Chem, 1997. **272**(25): p. 15986-92.
91. Brindley, D.N. and D.W. Waggoner, *Phosphatidate phosphohydrolase and signal transduction.* Chem Phys Lipids, 1996. **80**(1-2): p. 45-57.
92. Sciorra, V.A. and A.J. Morris, *Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells.* Mol Biol Cell, 1999. **10**(11): p. 3863-76.
93. Fourcade, O., et al., *Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells.* Cell, 1995. **80**(6): p. 919-27.
94. Jenkins, G.M. and M.A. Frohman, *Phospholipase D: a lipid centric review.* Cellular and Molecular Life Sciences, 2005. **62**: p. 2305-2316.
95. Athenstaedt, K. and G. Daum, *Phosphatidic acid, a key intermediate in lipid metabolism.* Eur J Biochem, 1999. **266**(1): p. 1-16.

96. Foster, D.A. and L. Xu, *Phospholipase D in cell proliferation and cancer.* Mol Cancer Res, 2003. **1**(11): p. 789-800.
97. Jenkins, G.H., P.L. Fisette, and R.A. Anderson, *Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid.* J Biol Chem, 1994. **269**(15): p. 11547-54.
98. Moritz, A., et al., *Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase.* J Biol Chem, 1992. **267**(11): p. 7207-10.
99. Rizzo, M.A., et al., *The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras.* J Biol Chem, 2000. **275**(31): p. 23911-8.
100. Fang, Y., et al., *Phosphatidic acid-mediated mitogenic activation of mTOR signaling.* Science, 2001. **294**(5548): p. 1942-5.
101. Song, J.G., L.M. Pfeffer, and D.A. Foster, *v-Src increases diacylglycerol levels via a type D phospholipase-mediated hydrolysis of phosphatidylcholine.* Mol Cell Biol, 1991. **11**(10): p. 4903-8.
102. Lu, Z., et al., *Tumor promotion by depleting cells of protein kinase C delta.* Mol Cell Biol, 1997. **17**(6): p. 3418-28.
103. Joseph, T., et al., *Transformation of cells overexpressing a tyrosine kinase by phospholipase D1 and D2.* Biochem Biophys Res Commun, 2001. **289**(5): p. 1019-24.
104. Lu, Z., et al., *Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3Y1 rat fibroblasts.* Mol Cell Biol, 2000. **20**(2): p. 462-7.
105. Buchanan, F.G., et al., *Requirement of phospholipase D1 activity in H-RasV12-induced transformation.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1638-42.
106. Min, D.S., et al., *Neoplastic transformation and tumorigenesis associated with overexpression of phospholipase D isozymes in cultured murine fibroblasts.* Carcinogenesis, 2001. **22**(10): p. 1641-7.
107. Jiang, Y.W., et al., *Phosphatidylcholine-specific phospholipase D activity is elevated in v-Fps-transformed cells.* Biochem Biophys Res Commun, 1994. **203**(2): p. 1195-2003.
108. Kasai, T., et al., *Increased activity of oleate-dependent type phospholipase D during actinomycin D-induced apoptosis in Jurkat T cells.* J Immunol, 1998. **161**(12): p. 6469-74.
109. Kang, J.H., I. Shin, and J.S. Han, *Changes of phospholipase D activity in TNF-alpha and anti-Fas/Apo1 monoclonal antibody induced apoptosis in HL-60 and A20 cells.* Exp Mol Med, 1998. **30**(1): p. 21-7.
110. Iwasaki-Bessho, Y., et al., *Decreased phospholipase D (PLD) activity in ceramide-induced apoptosis of human keratinocyte cell line HaCaT.* J Invest Dermatol, 1998. **110**(4): p. 376-82.
111. Chen, J.S., et al., *Regulation of phospholipase D activity and ceramide production in daunorubicin-induced apoptosis in A-431 cells.* Biochim Biophys Acta, 2000. **1488**(3): p. 219-32.
112. Kim, K.O., et al., *Anti-apoptotic role of phospholipase D isozymes in the glutamate-induced cell death.* Exp Mol Med, 2003. **35**(1): p. 38-45.

113. Zhong, M., et al., *Phospholipase D prevents apoptosis in v-Src-transformed rat fibroblasts and MDA-MB-231 breast cancer cells.* Biochem Biophys Res Commun, 2003. **302**(3): p. 615-9.
114. Kassis, J., et al., *Tumor invasion as dysregulated cell motility.* Semin Cancer Biol, 2001. **11**(2): p. 105-17.
115. Cross, M.J., et al., *Stimulation of actin stress fibre formation mediated by activation of phospholipase D.* Curr Biol, 1996. **6**(5): p. 588-97.
116. Kam, Y. and J.H. Exton, *Phospholipase D activity is required for actin stress fiber formation in fibroblasts.* Mol Cell Biol, 2001. **21**(12): p. 4055-66.
117. Ha, K.S., E.J. Yeo, and J.H. Exton, *Lysophosphatidic acid activation of phosphatidylcholine-hydrolysing phospholipase D and actin polymerization by a pertussis toxin-sensitive mechanism.* Biochem J, 1994. **303 (Pt 1)**: p. 55-9.
118. Aguirre Ghiso, J.A., et al., *A phospholipase D and protein kinase C inhibitor blocks the spreading of murine mammary adenocarcinoma cells altering f-actin and beta1-integrin point contact distribution.* Int J Cancer, 1997. **71**(5): p. 881-90.
119. Shen, Y., Y. Zheng, and D.A. Foster, *Phospholipase D2 stimulates cell protrusion in v-Src-transformed cells.* Biochem Biophys Res Commun, 2002. **293**(1): p. 201-6.
120. Hall, A., *Rho GTPases and the actin cytoskeleton.* Science, 1998. **279**(5350): p. 509-14.
121. Singer, W.D., et al., *Resolved phospholipase D activity is modulated by cytosolic factors other than Arf.* J Biol Chem, 1995. **270**(25): p. 14944-50.
122. Overall, C.M. and C. Lopez-Otin, *Strategies for MMP inhibition in cancer: innovations for the post-trial era.* Nat Rev Cancer, 2002. **2**(9): p. 657-72.
123. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: diversity and escape mechanisms.* Nat Rev Cancer, 2003. **3**(5): p. 362-74.
124. Aguirre-Ghiso, J.A., et al., *RalA requirement for v-Src- and v-Ras-induced tumorigenicity and overproduction of urokinase-type plasminogen activator: involvement of metalloproteases.* Oncogene, 1999. **18**(33): p. 4718-25.
125. Reich, R., M. Blumenthal, and M. Liscovitch, *Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells.* Clin Exp Metastasis, 1995. **13**(2): p. 134-40.
126. Williger, B.T., W.T. Ho, and J.H. Exton, *Phospholipase D mediates matrix metalloproteinase-9 secretion in phorbol ester-stimulated human fibrosarcoma cells.* J Biol Chem, 1999. **274**(2): p. 735-8.
127. Williger, B.T., et al., *Release of gelatinase A (matrix metalloproteinase 2) induced by photolysis of caged phosphatidic acid in HT 1080 metastatic fibrosarcoma cells.* J Biol Chem, 1995. **270**(50): p. 29656-9.
128. Imamura, F., et al., *Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D.* Biochem Biophys Res Commun, 1993. **193**(2): p. 497-503.
129. Pai, J.K., et al., *Novel ketoepoxides block phospholipase D activation and tumor cell invasion.* Anticancer Drug Des, 1994. **9**(4): p. 363-72.
130. Zheng, Y., et al., *Phospholipase D couples survival and migration signals in stress response of human cancer cells.* J Biol Chem, 2006. **281**(23): p. 15862-8.

131. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
132. Uchida, N., et al., *Increased phospholipase D activity in human breast cancer.* J Cancer Res Clin Oncol, 1997. **123**(5): p. 280-5.
133. Yoshida, M., et al., *Enhanced levels of oleate-dependent and Arf-dependent phospholipase D isoforms in experimental colon cancer.* Oncol Res, 1998. **10**(8): p. 399-406.
134. Uchida, N., S. Okamura, and H. Kuwano, *Phospholipase D activity in human gastric carcinoma.* Anticancer Res, 1999. **19**(1B): p. 671-5.
135. Zhao, Y., et al., *Increased activity and intranuclear expression of phospholipase D2 in human renal cancer.* Biochem Biophys Res Commun, 2000. **278**(1): p. 140-3.
136. Chen, Y., Y. Zheng, and D.A. Foster, *Phospholipase D confers rapamycin resistance in human breast cancer cells.* Oncogene, 2003. **22**(25): p. 3937-42.
137. Yarden, Y., *The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities.* Eur J Cancer, 2001. **37 Suppl 4**: p. S3-8.
138. Cohen, S., *Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal.* J Biol Chem, 1962. **237**: p. 1555-62.
139. Gregory, H., *Isolation and structure of urogastrone and its relationship to epidermal growth factor.* Nature, 1975. **257**(5524): p. 325-7.
140. Cohen, S. and G. Carpenter, *Human epidermal growth factor: isolation and chemical and biological properties.* Proc Natl Acad Sci U S A, 1975. **72**(4): p. 1317-21.
141. Cohen, S., G. Carpenter, and L. King, Jr., *Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity.* J Biol Chem, 1980. **255**(10): p. 4834-42.
142. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.
143. Singh, A.B. and R.C. Harris, *Autocrine, paracrine and juxtacrine signaling by EGFR ligands.* Cell Signal, 2005. **17**(10): p. 1183-93.
144. Thompson, D.M. and G.N. Gill, *The EGF receptor: structure, regulation and potential role in malignancy.* Cancer Surv, 1985. **4**(4): p. 767-88.
145. Lax, I., et al., *Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules.* EMBO J, 1989. **8**(2): p. 421-7.
146. Wells, A., *EGF receptor.* The International Journal of Biochemistry & Cell Biology, 1999. **31**(6): p. 637-643.
147. Li, X., et al., *Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway.* EMBO J, 1998. **17**(9): p. 2574-83.
148. Sun, H., et al., *The juxtamembrane, cytosolic region of the epidermal growth factor receptor is involved in association with alpha-subunit of Gs.* J Biol Chem, 1997. **272**(9): p. 5413-20.

149. Daub, H., et al., *Signal characteristics of G protein-transactivated EGF receptor.* EMBO J, 1997. **16**(23): p. 7032-44.
150. Carpenter, G. and S. Cohen, *Epidermal growth factor.* J Biol Chem, 1990. **265**(14): p. 7709-12.
151. Bertics, P.J., et al., *Alteration of epidermal growth factor receptor activity by mutation of its primary carboxyl-terminal site of tyrosine self-phosphorylation.* J Biol Chem, 1988. **263**(8): p. 3610-7.
152. Helin, K., et al., *The biological activity of the human epidermal growth factor receptor is positively regulated by its C-terminal tyrosines.* Oncogene, 1991. **6**(5): p. 825-32.
153. Herbst, R.S., *Review of epidermal growth factor receptor biology.* Int J Radiat Oncol Biol Phys, 2004. **59**(2 Suppl): p. 21-6.
154. Harris, R.C., E. Chung, and R.J. Coffey, *EGF receptor ligands.* Exp Cell Res, 2003. **284**(1): p. 2-13.
155. Lee, D.C., et al., *TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase.* Ann N Y Acad Sci, 2003. **995**: p. 22-38.
156. Citri, A. and Y. Yarden, *EGF-ERBB signalling: towards the systems level.* Nat Rev Mol Cell Biol, 2006. **7**(7): p. 505-16.
157. Levkowitz, G., et al., *Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1.* Mol Cell, 1999. **4**(6): p. 1029-40.
158. Levkowitz, G., et al., *c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor.* Genes Dev, 1998. **12**(23): p. 3663-74.
159. Lenferink, A.E., et al., *Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers.* EMBO J, 1998. **17**(12): p. 3385-97.
160. Reddy, C.C., A. Wells, and D.A. Lauffenburger, *Receptor-mediated effects on ligand availability influence relative mitogenic potencies of epidermal growth factor and transforming growth factor alpha.* J Cell Physiol, 1996. **166**(3): p. 512-22.
161. Voldborg, B.R., et al., *Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials.* Ann Oncol, 1997. **8**(12): p. 1197-206.
162. Vojtek, A.B. and C.J. Der, *Increasing complexity of the Ras signaling pathway.* J Biol Chem, 1998. **273**(32): p. 19925-8.
163. Weihua, Z., et al., *Survival of cancer cells is maintained by EGFR independent of its kinase activity.* Cancer Cell, 2008. **13**(5): p. 385-93.
164. Park, J.B., et al., *Regulation of phospholipase D2 by GTP-dependent interaction with dynamin.* Adv Enzyme Regul, 2004. **44**: p. 249-64.
165. Lee, C.S., et al., *The phox homology domain of phospholipase D activates dynamin GTPase activity and accelerates EGFR endocytosis.* Nat Cell Biol, 2006. **8**(5): p. 477-84.
166. Mazie, A.R., et al., *Epithelial cell motility is triggered by activation of the EGF receptor through phosphatidic acid signaling.* J Cell Sci, 2006. **119**(Pt 8): p. 1645-54.

167. Fischer, O.M., et al., *EGFR signal transactivation in cancer cells.* Biochem Soc Trans, 2003. **31**(Pt 6): p. 1203-8.
168. Salomon, D.S., et al., *Epidermal growth factor-related peptides and their receptors in human malignancies.* Crit Rev Oncol Hematol, 1995. **19**(3): p. 183-232.
169. Carpenter, G. and S. Cohen, *Epidermal growth factor.* Annu Rev Biochem, 1979. **48**: p. 193-216.
170. Ennis, B.W., M.E. Lippman, and R.B. Dickson, *The EGF receptor system as a target for antitumor therapy.* Cancer Invest, 1991. **9**(5): p. 553-62.
171. Kondapaka, S.B., R. Fridman, and K.B. Reddy, *Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells.* Int J Cancer, 1997. **70**(6): p. 722-6.
172. Newby, J.C., et al., *Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer.* Clin Cancer Res, 1997. **3**(9): p. 1643-51.
173. Sartor, C.I., *Biological modifiers as potential radiosensitizers: targeting the epidermal growth factor receptor family.* Semin Oncol, 2000. **27**(6 Suppl 11): p. 15-20; discussion 92-100.
174. Chen, X., T.K. Yeung, and Z. Wang, *Enhanced drug resistance in cells coexpressing ErbB2 with EGF receptor or ErbB3.* Biochem Biophys Res Commun, 2000. **277**(3): p. 757-63.
175. Fischer-Colbrie, J., et al., *EGFR and steroid receptors in ovarian carcinoma: comparison with prognostic parameters and outcome of patients.* Anticancer Res, 1997. **17**(1B): p. 613-9.
176. Brabender, J., et al., *Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer Is correlated with survival.* Clin Cancer Res, 2001. **7**(7): p. 1850-5.
177. Arteaga, C.L., *Epidermal growth factor receptor dependence in human tumors: more than just expression?* Oncologist, 2002. **7 Suppl 4**: p. 31-9.
178. Baselga, J., *New therapeutic agents targeting the epidermal growth factor receptor.* J Clin Oncol, 2000. **18**(21 Suppl): p. 54S-9S.
179. Rowinsky, E.K., *The pursuit of optimal outcomes in cancer therapy in a new age of rationally designed target-based anticancer agents.* Drugs, 2000. **60 Suppl 1**: p. 1-14; discussion 41-2.
180. Karnoub, A.E. and R.A. Weinberg, *Ras oncogenes: split personalities.* Nat Rev Mol Cell Biol, 2008. **9**(7): p. 517-31.
181. DeFeo, D., et al., *Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus.* Proc Natl Acad Sci U S A, 1981. **78**(6): p. 3328-32.
182. Ellis, R.W., et al., *Mouse cells contain two distinct ras gene mRNA species that can be translated into a p21 onc protein.* Mol Cell Biol, 1982. **2**(11): p. 1339-45.
183. Chang, E.H., et al., *Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses.* Proc Natl Acad Sci U S A, 1982. **79**(16): p. 4848-52.
184. Lowy, D.R. and B.M. Willumsen, *Function and regulation of ras.* Annu Rev Biochem, 1993. **62**: p. 851-91.

185. Hancock, J.F., *Ras proteins: different signals from different locations.* Nat Rev Mol Cell Biol, 2003. **4**(5): p. 373-84.
186. Grand, R.J. and D. Owen, *The biochemistry of ras p21.* Biochem J, 1991. **279 (Pt 3)**: p. 609-31.
187. Jaumot, M., et al., *The linker domain of the Ha-Ras hypervariable region regulates interactions with exchange factors, Raf-1 and phosphoinositide 3-kinase.* J Biol Chem, 2002. **277**(1): p. 272-8.
188. Silvius, J.R., *Mechanisms of Ras protein targeting in mammalian cells.* J Membr Biol, 2002. **190**(2): p. 83-92.
189. Wolfman, A., *Ras isoform-specific signaling: location, location, location.* Sci STKE, 2001. **2001**(96): p. PE2.
190. Shields, J.M., et al., *Understanding Ras: 'it ain't over 'til it's over'.* Trends Cell Biol, 2000. **10**(4): p. 147-54.
191. Cai, D., et al., *Phospholipase D1 corrects impaired betaAPP trafficking and neurite outgrowth in familial Alzheimer's disease-linked presenilin-1 mutant neurons.* Proc Natl Acad Sci U S A, 2006. **103**(6): p. 1936-40.
192. Zheng, Y. and L.A. Quilliam, *Activation of the Ras superfamily of small GTPases. Workshop on exchange factors.* EMBO Rep, 2003. **4**(5): p. 463-8.
193. Satoh, T., M. Nakafuku, and Y. Kaziro, *Function of Ras as a molecular switch in signal transduction.* J Biol Chem, 1992. **267**(34): p. 24149-52.
194. Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-binding proteins.* Physiol Rev, 2001. **81**(1): p. 153-208.
195. Satoh, T., et al., *Platelet-derived growth factor stimulates formation of active p21ras.GTP complex in Swiss mouse 3T3 cells.* Proc Natl Acad Sci U S A, 1990. **87**(15): p. 5993-7.
196. Satoh, T., et al., *Accumulation of p21ras.GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity.* Proc Natl Acad Sci U S A, 1990. **87**(20): p. 7926-9.
197. Bar-Sagi, D., *The Sos (Son of sevenless) protein.* Trends Endocrinol Metab, 1994. **5**(4): p. 165-9.
198. Gibbs, J.B., et al., *Purification of ras GTPase activating protein from bovine brain.* Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5026-30.
199. Moodie, S.A., et al., *Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase.* Science, 1993. **260**(5114): p. 1658-61.
200. Warne, P.H., P.R. Viciana, and J. Downward, *Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro.* Nature, 1993. **364**(6435): p. 352-5.
201. Zhang, X.F., et al., *Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1.* Nature, 1993. **364**(6435): p. 308-13.
202. Vojtek, A.B., S.M. Hollenberg, and J.A. Cooper, *Mammalian Ras interacts directly with the serine/threonine kinase Raf.* Cell, 1993. **74**(1): p. 205-14.
203. Reuter, C.W., M.A. Morgan, and L. Bergmann, *Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies?* Blood, 2000. **96**(5): p. 1655-69.
204. Hofer, F., et al., *Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator.* Proc Natl Acad Sci U S A, 1994. **91**(23): p. 11089-93.

205. Wolthuis, R.M., et al., *Ras-dependent activation of the small GTPase Ral.* Curr Biol, 1998. **8**(8): p. 471-4.
206. Rodriguez-Viciana, P., et al., *Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras.* Cell, 1997. **89**(3): p. 457-67.
207. Khwaja, A., et al., *Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway.* EMBO J, 1997. **16**(10): p. 2783-93.
208. Hamad, N.M., et al., *Distinct requirements for Ras oncogenesis in human versus mouse cells.* Genes Dev, 2002. **16**(16): p. 2045-57.
209. Boman, A.L. and R.A. Kahn, *Arf proteins: the membrane traffic police?* Trends Biochem Sci, 1995. **20**(4): p. 147-50.
210. Xu, L., et al., *Elevated phospholipase D activity in H-Ras- but not K-Ras-transformed cells by the synergistic action of RalA and ARF6.* Mol Cell Biol, 2003. **23**(2): p. 645-54.
211. Jiang, H., et al., *Ras mediates the activation of phospholipase D by v-Src.* J Biol Chem, 1995. **270**(11): p. 6006-9.
212. Jiang, H., et al., *Involvement of Ral GTPase in v-Src-induced phospholipase D activation.* Nature, 1995. **378**(6555): p. 409-12.
213. Shi, M., et al., *Phospholipase D provides a survival signal in human cancer cells with activated H-Ras or K-Ras.* Cancer Lett, 2007. **258**(2): p. 268-75.
214. Reddy, E.P., et al., *A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene.* Nature, 1982. **300**(5888): p. 149-52.
215. Taparowsky, E., et al., *Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change.* Nature, 1982. **300**(5894): p. 762-5.
216. Sprang, S.R., *G protein mechanisms: insights from structural analysis.* Annu Rev Biochem, 1997. **66**: p. 639-78.
217. Hirai, H., et al., *Activation of the c-K-ras oncogene in a human pancreas carcinoma.* Biochem Biophys Res Commun, 1985. **127**(1): p. 168-74.
218. Hand, P.H., et al., *Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas.* Proc Natl Acad Sci U S A, 1984. **81**(16): p. 5227-31.
219. Fujita, J., et al., *Ha-ras oncogenes are activated by somatic alterations in human urinary tract tumours.* Nature, 1984. **309**(5967): p. 464-6.
220. Bos, J.L., et al., *Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia.* Nature, 1985. **315**(6022): p. 726-30.
221. Padua, R.A., N.C. Barrass, and G.A. Currie, *Activation of N-ras in a human melanoma cell line.* Mol Cell Biol, 1985. **5**(3): p. 582-5.
222. Hardie, D.G., *Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status.* Endocrinology, 2003. **144**(12): p. 5179-83.
223. Hardie, D.G. and S.A. Hawley, *AMP-activated protein kinase: the energy charge hypothesis revisited.* Bioessays, 2001. **23**(12): p. 1112-9.
224. Sim, A.T. and D.G. Hardie, *The low activity of acetyl-CoA carboxylase in basal and glucagon-stimulated hepatocytes is due to phosphorylation by the AMP-*

activated protein kinase and not cyclic AMP-dependent protein kinase. FEBS Lett, 1988. **233**(2): p. 294-8.
225. Stapleton, D., et al., *AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location.* FEBS Lett, 1997. **409**(3): p. 452-6.
226. Hardie, D.G., et al., *Management of cellular energy by the AMP-activated protein kinase system.* FEBS Lett, 2003. **546**(1): p. 113-20.
227. Carling, D., *The AMP-activated protein kinase cascade--a unifying system for energy control.* Trends Biochem Sci, 2004. **29**(1): p. 18-24.
228. Thornton, C., M.A. Snowden, and D. Carling, *Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle.* J Biol Chem, 1998. **273**(20): p. 12443-50.
229. Cheung, P.C., et al., *Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding.* Biochem J, 2000. **346 Pt 3**: p. 659-69.
230. Crute, B.E., et al., *Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase.* J Biol Chem, 1998. **273**(52): p. 35347-54.
231. Hudson, E.R., et al., *A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias.* Curr Biol, 2003. **13**(10): p. 861-6.
232. Polekhina, G., et al., *AMPK beta subunit targets metabolic stress sensing to glycogen.* Curr Biol, 2003. **13**(10): p. 867-71.
233. Mitchelhill, K.I., et al., *Posttranslational modifications of the 5'-AMP-activated protein kinase beta1 subunit.* J Biol Chem, 1997. **272**(39): p. 24475-9.
234. Warden, S.M., et al., *Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization.* Biochem J, 2001. **354**(Pt 2): p. 275-83.
235. Corton, J.M., J.G. Gillespie, and D.G. Hardie, *Role of the AMP-activated protein kinase in the cellular stress response.* Curr Biol, 1994. **4**(4): p. 315-24.
236. Marsin, A.S., et al., *Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia.* Curr Biol, 2000. **10**(20): p. 1247-55.
237. Kudo, N., et al., *High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase.* J Biol Chem, 1995. **270**(29): p. 17513-20.
238. Salt, I.P., et al., *AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release.* Biochem J, 1998. **335 (Pt 3)**: p. 533-9.
239. Winder, W.W. and D.G. Hardie, *Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise.* Am J Physiol, 1996. **270**(2 Pt 1): p. E299-304.
240. Fujii, N., et al., *Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle.* Biochem Biophys Res Commun, 2000. **273**(3): p. 1150-5.
241. Hawley, S.A., et al., *Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it*

phosphorylates AMP-activated protein kinase. J Biol Chem, 1996. **271**(44): p. 27879-87.
242. Stein, S.C., et al., *The regulation of AMP-activated protein kinase by phosphorylation.* Biochem J, 2000. **345 Pt 3**: p. 437-43.
243. Hardie, D.G., et al., *AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge.* Biochem J, 1999. **338 (Pt 3)**: p. 717-22.
244. Hadad, S.M., S. Fleming, and A.M. Thompson, *Targeting AMPK: A new therapeutic opportunity in breast cancer.* Crit Rev Oncol Hematol, 2008. **67**(1): p. 1-7.
245. Alessi, D.R., K. Sakamoto, and J.R. Bayascas, *LKB1-dependent signaling pathways.* Annu Rev Biochem, 2006. **75**: p. 137-63.
246. Hemminki, A., *The molecular basis and clinical aspects of Peutz-Jeghers syndrome.* Cell Mol Life Sci, 1999. **55**(5): p. 735-50.
247. Giardiello, F.M., et al., *Very high risk of cancer in familial Peutz-Jeghers syndrome.* Gastroenterology, 2000. **119**(6): p. 1447-53.
248. Sanchez-Cespedes, M., et al., *Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung.* Cancer Res, 2002. **62**(13): p. 3659-62.
249. Ji, H., et al., *LKB1 modulates lung cancer differentiation and metastasis.* Nature, 2007. **448**(7155): p. 807-10.
250. Hawley, S.A., et al., *Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade.* J Biol, 2003. **2**(4): p. 28.
251. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3329-35.
252. Woods, A., et al., *LKB1 is the upstream kinase in the AMP-activated protein kinase cascade.* Curr Biol, 2003. **13**(22): p. 2004-8.
253. Sakamoto, K., et al., *Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction.* EMBO J, 2005. **24**(10): p. 1810-20.
254. Towler, M.C. and D.G. Hardie, *AMP-activated protein kinase in metabolic control and insulin signaling.* Circ Res, 2007. **100**(3): p. 328-41.
255. Shen, Q.W., et al., *Ca2+/calmodulin-dependent protein kinase kinase is involved in AMP-activated protein kinase activation by alpha-lipoic acid in C2C12 myotubes.* Am J Physiol Cell Physiol, 2007. **293**(4): p. C1395-403.
256. Wallimann, T., *Bioenergetics. Dissecting the role of creatine kinase.* Curr Biol, 1994. **4**(1): p. 42-6.
257. Schlattner, U., M. Tokarska-Schlattner, and T. Wallimann, *Mitochondrial creatine kinase in human health and disease.* Biochim Biophys Acta, 2006. **1762**(2): p. 164-80.
258. Ponticos, M., et al., *Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle.* EMBO J, 1998. **17**(6): p. 1688-99.
259. Wojtaszewski, J.F., et al., *Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle.* Diabetes, 2002. **51**(2): p. 284-92.

260. Wojtaszewski, J.F., et al., *Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle.* Am J Physiol Endocrinol Metab, 2003. **284**(4): p. E813-22.
261. Corton, J.M., et al., *5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells?* Eur J Biochem, 1995. **229**(2): p. 558-65.
262. Bolster, D.R., et al., *AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling.* J Biol Chem, 2002. **277**(27): p. 23977-80.
263. Foretz, M., et al., *AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes.* J Biol Chem, 1998. **273**(24): p. 14767-71.
264. Clarke, P.R. and D.G. Hardie, *Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase in vitro and in intact rat liver.* EMBO J, 1990. **9**(8): p. 2439-46.
265. Fryer, L.G., et al., *Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells.* Biochem J, 2002. **363**(Pt 1): p. 167-74.
266. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes Dev, 2004. **18**(16): p. 1926-45.
267. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR signaling in growth and metabolism.* Cell, 2006. **124**(3): p. 471-84.
268. Krause, U., L. Bertrand, and L. Hue, *Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes.* Eur J Biochem, 2002. **269**(15): p. 3751-9.
269. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): p. 577-90.
270. Gwinn, D.M., et al., *AMPK phosphorylation of raptor mediates a metabolic checkpoint.* Mol Cell, 2008. **30**(2): p. 214-26.
271. Winder, W.W. and D.G. Hardie, *AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes.* Am J Physiol, 1999. **277**(1 Pt 1): p. E1-10.
272. Bergeron, R., et al., *Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats.* Diabetes, 2001. **50**(5): p. 1076-82.
273. Buhl, E.S., et al., *Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of the insulin resistance syndrome.* Diabetes, 2002. **51**(7): p. 2199-206.
274. Winder, W.W., et al., *Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle.* J Appl Physiol, 2000. **88**(6): p. 2219-26.
275. Fryer, L.G., A. Parbu-Patel, and D. Carling, *The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways.* J Biol Chem, 2002. **277**(28): p. 25226-32.
276. Goodyear, L.J. and B.B. Kahn, *Exercise, glucose transport, and insulin sensitivity.* Annu Rev Med, 1998. **49**: p. 235-61.

277. Luo, Z., et al., *AMPK, the metabolic syndrome and cancer.* Trends Pharmacol Sci, 2005. **26**(2): p. 69-76.
278. Xiang, X., et al., *AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms.* Biochem Biophys Res Commun, 2004. **321**(1): p. 161-7.
279. Saitoh, M., et al., *Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase.* Biochem Pharmacol, 2004. **67**(10): p. 2005-11.
280. Li, J., et al., *AMPK-beta1 subunit is a p53-independent stress responsive protein that inhibits tumor cell growth upon forced expression.* Carcinogenesis, 2003. **24**(5): p. 827-34.
281. Blazquez, C., et al., *The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes.* FEBS Lett, 2001. **489**(2-3): p. 149-53.
282. Durante, P., et al., *Apoptosis induced by growth factor withdrawal in fibroblasts overproducing fructose 2,6-bisphosphate.* FEBS Lett, 1999. **448**(2-3): p. 239-43.
283. Kato, K., et al., *Critical roles of AMP-activated protein kinase in constitutive tolerance of cancer cells to nutrient deprivation and tumor formation.* Oncogene, 2002. **21**(39): p. 6082-90.
284. D'Souza-Schorey, C., et al., *A regulatory role for ARF6 in receptor-mediated endocytosis.* Science, 1995. **267**(5201): p. 1175-8.
285. Luo, J.Q., et al., *Functional association between Arf and RalA in active phospholipase D complex.* Proc Natl Acad Sci U S A, 1998. **95**(7): p. 3632-7.
286. Anderson, N.G., et al., *ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression.* Int J Cancer, 2001. **94**(6): p. 774-82.
287. Kozma, S.C., et al., *The human c-Kirsten ras gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231.* Nucleic Acids Res, 1987. **15**(15): p. 5963-71.
288. Shimizu, K., et al., *Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1.* Nature, 1983. **304**(5926): p. 497-500.
289. Moasser, M.M., et al., *The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells.* Cancer Res, 2001. **61**(19): p. 7184-8.
290. Rusnak, D.W., et al., *Assessment of epidermal growth factor receptor (EGFR, ErbB1) and HER2 (ErbB2) protein expression levels and response to lapatinib (Tykerb, GW572016) in an expanded panel of human normal and tumour cell lines.* Cell Prolif, 2007. **40**(4): p. 580-94.
291. Feig, L.A., *Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases.* Nat Cell Biol, 1999. **1**(2): p. E25-7.
292. Fedi, P., S.R. Tronick, and S.A. Aaronson, *Growth Factors. In Cancer Medicine*, ed. J.F. Holland, et al. 1997, Baltimore, MD: Wiliiams and Wilkins.
293. Medema, R.H. and J.L. Bos, *The role of p21ras in receptor tyrosine kinase signaling.* Crit Rev Oncog, 1993. **4**(6): p. 615-61.

294. Kim, J.H., et al., *Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA.* FEBS Lett, 1998. **430**(3): p. 231-5.
295. Esumi, T., et al., *Efficient synthesis and structure-activity relationship of honokiol, a neurotrophic biphenyl-type neolignan.* Bioorg Med Chem Lett, 2004. **14**(10): p. 2621-5.
296. Clark, A.M., F.S. El-Feraly, and W.S. Li, *Antimicrobial activity of phenolic constituents of Magnolia grandiflora L.* J Pharm Sci, 1981. **70**(8): p. 951-2.
297. Tsai, T.H. and C.F. Chen, *Identification and determination of honokiol and magnolol from Magnolis officinalis by high-performance liquid chromatography with photodiode-array UV detection.* J of Chromatography A, 1992. **598**: p. 143-146.
298. Teng, C.M., et al., *Two antiplatelet agents from Magnolia officinalis.* Thromb Res, 1988. **50**(6): p. 757-65.
299. Watanabe, K., et al., *Pharmacological properties of magnolol and honokiol extracted from Magnolia officinalis: central depressant effects.* Planta Med, 1983. **49**(2): p. 103-8.
300. Chen, F., et al., *Honokiol: a potent chemotherapy candidate for human colorectal carcinoma.* World J Gastroenterol, 2004. **10**(23): p. 3459-63.
301. Yang, S.E., et al., *Down-modulation of Bcl-XL, release of cytochrome c and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells.* Biochem Pharmacol, 2002. **63**(9): p. 1641-51.
302. Konoshima, T., et al., *Studies on inhibitors of skin tumor promotion, IX. Neolignans from Magnolia officinalis.* J Nat Prod, 1991. **54**(3): p. 816-22.
303. Bai, X., et al., *Honokiol, a small molecular weight natural product, inhibits angiogenesis in vitro and tumor growth in vivo.* J Biol Chem, 2003. **278**(37): p. 35501-7.
304. Battle, T.E., J. Arbiser, and D.A. Frank, *The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells.* Blood, 2005. **106**(2): p. 690-7.
305. Ishitsuka, K., et al., *Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis.* Blood, 2005. **106**(5): p. 1794-800.
306. Wolf, I., et al., *Honokiol, a natural biphenyl, inhibits in vitro and in vivo growth of breast cancer through induction of apoptosis and cell cycle arrest.* Int J Oncol, 2007. **30**(6): p. 1529-37.
307. Xu, D., Q. Lu, and X. Hu, *Down-regulation of P-glycoprotein expression in MDR breast cancer cell MCF-7/ADR by honokiol.* Cancer Lett, 2006. **243**(2): p. 274-80.
308. Mukherjee, P., et al., *Differential effects of energy stress on AMPK phosphorylation and apoptosis in experimental brain tumor and normal brain.* Mol Cancer, 2008. **7**: p. 37.
309. Laderoute, K.R., et al., *5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments.* Mol Cell Biol, 2006. **26**(14): p. 5336-47.
310. Hahn-Windgassen, A., et al., *Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity.* J Biol Chem, 2005. **280**(37): p. 32081-9.

311. Chen, H.C., et al., *Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-stimulated glucose transport.* J Biol Chem, 2002. **277**(26): p. 23554-62.
312. Proud, C.G., *The multifaceted role of mTOR in cellular stress responses.* DNA Repair (Amst), 2004. **3**(8-9): p. 927-34.
313. Chen, J. and Y. Fang, *A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling.* Biochem Pharmacol, 2002. **64**(7): p. 1071-7.
314. Garcia, A., et al., *Honokiol suppresses survival signals mediated by Ras-dependent phospholipase D activity in human cancer cells.* Clin Cancer Res, 2008. **14**(13): p. 4267-74.