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Role of Lipins in Regulating Phospholipase D Signalling

by

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ABSTRACT

A potential therapeutic target in cancer is phospholipase D (PLD), which converts phosphatidylcholine into phosphatidic acid, a lipid second messenger in cell signalling. The lipid phosphate phosphatases are considered to convert PLD-generated phosphatidic acid to diacylglycerol. However, the lipid phosphate phosphatases are integral membrane proteins with catalytic sites on the extracellular or lumenal sides of membranes whereas phosphatidic acid accumulates on the cytosolic leaflet of membranes. The other enzymes that dephosphorylate phosphatidic acid, the lipins, are cytosolic proteins that translocate to membranes. This project investigated whether lipin-1 and lipin-2 regulate PLD signalling. These results show that phosphatidic acid accumulation in breast cancer cells resulted from PLD1 and PLD2 activation and activity of calcium-dependent diacylglycerol kinases. Depletion of lipin-1 did not affect PLD-dependent phosphatidic acid accumulation in fibroblasts. This work provides the first evidence that increased phosphatidic acid phosphatase activity of lipins is associated with membranes in response to PLD stimulation.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DAG	diacylglycerol
DGK	diacylglycerol kinase
DMSO	dimethyl sulfoxide
DHA	docosahexaenoic acid
DSP	dimethylsuccinimydylpropionate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGTA	ethyleneglycoltetraacetic acid
ERK1/2	Extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
FBS-C	charcoal-treated fetal bovine serum
Fld	fatty liver dystrophy
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GEF	guanine nucleotide exchange factor
GPAT	glycerol-3-phosphate acyltransferase
GPCR	G-protein coupled receptor
GSK-3	glycogen synthase kinase-3
HBS	HEPES-buffered saline
IP	immunoprecipitation
$iPLA_2$	Ca^{2+}-independent phospholipase A_2

LPA	lysophosphatidic acid
LPAAT	lysophosphatidate acyltransferase
LPP	lipid phosphate phosphatase
LDH	lactate dehydrogenase
MAG	monoacylglycerol
MEF	Mouse embryonic fibroblast
MEK	mitogen-activated protein kinase kinase
mRNA	messenger ribonucleic acid
NEM	N-ethylmaleimide
NF-κB	Nuclear Factor κ-B
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PBS	phosphate buffered saline
PB	phosphatidylbutanol
PC	Phosphatidylcholine
PKCε	Protein kinase Cε
$PLA_{1/2}$	Phospholipase $A_{1/2}$
PLD	Phospholipase D
PLC	Phospholipase C
PFA	paraformaldehyde
PMA	phorbol myristate acetate
PP-1	protein phosphatase-1
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
S1P	sphingosine-1-phosphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

siRNA	small interfering RNA
TAG	triacylglycerol
TDZ	thiazolidinedione
TLC	thin layer chromatography
TLR	toll-like receptor
TNFα	transcription factor α

CHAPTER 1: INTRODUCTION

1.1. Overview of Thesis

Phospholipase D (PLD) enzymes are becoming recognized as playing an important role in regulating cell survival, migration and tumorigenesis. The PLD enzymes generate intracellular phosphatidic acid (PA) which acts as a second messenger in cell signalling pathways that promote cell survival. PA can be converted to diacylgycerol (DAG), which is also a second messenger required in inflammatory signalling and several other signalling pathways. The degradation of PA produced by PLD is currently not well understood. There are two families of phosphatidic acid phosphatase (PAP) enzymes which produce DAG, lipid phosphate phosphatases (LPPs) and lipins. Classically, the LPPs have been considered to degrade the PA produced by the PLD pathway. The LPPs play several roles in regulating intracellular and extracellular signalling. However, insights into the structure and signalling functions of LPPs have raised questions about how LPPs could hydrolyze intracellular PA formed by the PLDs. The goal of this thesis is to obtain experimental evidence for or against a role for the lipin family members as PAP enzymes in the PLD pathway. The lipins play a major role in glycerolipid synthesis in addition to their roles in regulating transcription and cell signalling. Evidence that will be reviewed in this Chapter shows how the structural and functional characteristics of lipins also support a role for lipins in regulating the PLD pathway.

1.2. Introduction to the Phospholipase D Pathway

1.2.1. Importance of the phospholipase D pathway in cell signalling

In the PLD pathway, PLD enzymes convert phosphatidylcholine (PC) to PA which can be degraded by a variety of enzymes as depicted in Figure 1.1. PLD-derived PA can be dephosphorylated by the PAP enzymes to form the neutral lipid DAG. The PAP enzymes regulate the ratio of PA to DAG. PA can be cleaved at its *sn-1* acyl chain by cytosolic phospholipase A_1 ($cPLA_1$) or at its *sn-2* acyl chain by cytosolic phospholipase A_2 ($cPLA_2$) to form lysophosphatidic acid (LPA) [1]. DAG can also be formed by phospholipase C (PLC) activity. PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to DAG and inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} are released [2]. DAG can activate protein kinase C (PKC) enzymes which in turn activate PLD as will be discussed in section 1.2.3. PA can also be formed by LPA acyltransferases (LPAATs). The signalling mechanisms to terminate DAG accumulation include metabolism of DAG to PA by DAG kinase (DGK) enzymes or conversion of DAG to monoacylglycerol (MAG) by diacylglycerol lipases [3,4].

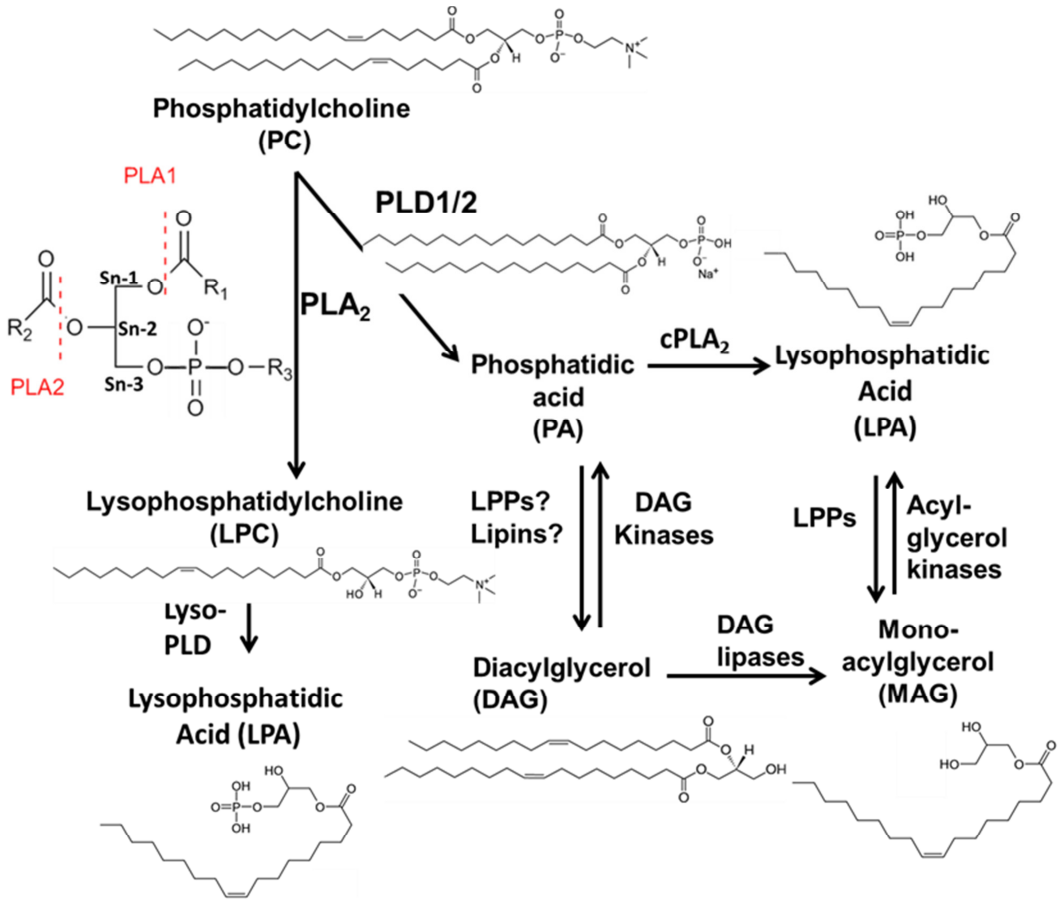


Figure 1.1. Metabolism and degradation of intracellular bioactive lipids. PLD1/2 converts PC into PA. Abbreviations and details about the enzymes are described in Section 1.2.1. The PLA_2 isoform is shown to convert PC to 1-acyl-2-lyso-PC and $cPLA_2$ (cytosolic phospholipase A_2) is shown to convert PA to 1-acyl-2-lyso-PA. The products formed by PLA_1 or $cLPA_1$ are not shown. Other abbreviations are as follows: LPPs (lipid phosphate phosphatases). Adapted from Mills *et al*. [5].

1.2.2. Roles of phosphatidic acid as a bioactive signalling lipid

PA is a bioactive lipid that activates cell signalling and cell migration. Several studies show that intracellular PA accumulation is associated with cell survival and proliferation [6–8]. PA is composed of a small negatively charged headgroup attached to a glycerol backbone and two fatty acyl chains (Fig. 1.1). PA binds to regions on intracellular target proteins, thereby targeting proteins to the membrane and regulating signalling outcomes. PA can function as a lipid anchor by directly binding to positively charged regions on effector proteins (polybasic motifs). Despite the numerous interactions of PA with proteins, no common PA binding domain has been recognized [9]. PA activates p42/44 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3-K) signalling, the mammalian Target of Rapamycin (mTOR), actin remodelling signalling pathways and stress fibre formation as will be discussed below.

The PA generated via PLD activity recruits the serine-threonine kinase Raf-1 to the plasma membrane, causing activation of the p42/44 MAPK pathway [10]. The domain of Raf-1 which binds to PA consists of a stretch of 4 positively charged residues which could participate in an electrostatic interaction with the negatively charged head group of PA and an association with the membrane via a hydrophobic subdomain which could dock Raf-1 at the membrane [11]. PA accumulation is also sufficient to recruit Raf-1 to the membrane without upstream receptor activation [12].

In response to PLD2 activation, accumulated PA binds with high affinity to the pleckstrin homology domain at the N-terminus of Sos and thereby recruits Sos to the plasma membrane [13]. At the membrane, Sos acts as a guanine nucleotide exchange factor (GEF) for Ras (Fig. 1.5) in a rate-limiting step that promotes the exchange of GDP for GTP by Ras [13]. PLD directly interacts with the growth factor receptor-bound protein 2 (Grb2) and COS7 cells in which Grb2 was depleted showed impaired activation of PLD2 [14]. PA can activate phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) and this is dependent on Sos and Raf recruitment to the membrane by PA [6,15].

Elevated PLD activity causes PA accumulation and PA inhibits the activity of the γ isoform of the catalytic subunit of protein-phosphatase-1 (PP-1cγ) [16]. PP-1cγ is a serine/threonine phosphatase that has been shown to bind to PA with high affinity [9]. Deletion mutagenesis studies showed that binding of PP-1cγ to PA is dependent upon residues 286-296 at the C-terminus of PP-1cγ [9].

The transcription factor c-Myc is activated by ERK1/2 signalling and PLD activity increases expression of the Myc onco-protein in MCF-7 breast cancer cells [17]. Elevated PLD activity decreases phosphorylation at Ser62 and Thr58 residues of Myc and inhibits degradation of Myc by the proteasome [17]. PLD inhibits the activity of protein phosphatase-2A (PP-2A) which dephosphorylates Myc. PP2A is regarded as a tumor suppressor because decreased PP2A expression was associated with increased signalling through c-Myc and the PI3K/Akt pathway. Increased Myc activity is associated with aggressive tumor phenotypes in breast cancer and poor clinical outcomes [17]. It has also been

proposed that PLD further promotes the stabilization of Myc through inhibition of PP-1 by increasing PA levels [18]. However, evidence demonstrating dephosphorylation of Myc by PP-1 is lacking.

PA can also activate signalling through intracellular sphingosine-1-phosphate (S1P) [19]. In addition, increased PLD activity has been shown to result in increased activation and translocation of sphingosine kinase-1 (SK-1) to membranes [20]. PLD-derived PA recruits sphingosine kinase-1 (SK-1) to membranes, where SK-1 converts sphingosine to S1P [21]. SK-1 binds to PA through a binding domain at the C-terminus [22]. S1P is a bioactive lipid that increases cell survival [21]. Intracellular S1P can be secreted from the cell by ABCC1 and ABCG2 transporters and can stimulate S1P receptors on the same cells (autocrine signalling) or from a nearby cell (paracrine signalling) [23]. Secreted S1P can also stimulate cell migration in endothelial cells [24] by activating S1P receptors on the cell surface. S1P binds to the $S1P_1$ receptor in a paracrine manner to regulate thymocyte motility and lymphocyte migration and distribution [25]. S1P from mast cells controls migration of mast cells [25]. S1P activates mast cells in an autocrine manner to secrete allergenic and pro-inflammatory signalling proteins [20]. SK-1 participates in autocrine signalling by associating with the $S1P_1$ receptor at lamellipodia in response to PDGF stimulation [26]. This interaction between SK-1 and the $S1P_1$ receptor promotes cell migration [26]. Phosphorylation of SK-1 by ERK1/2 is also required to activate SK-1 and promote its translocation to membrane compartments where it can phosphorylate sphingosine [27].

PLD activity is required for cell adhesion and PA derived from PLD activation increases the binding affinity of cells during attachment in culture [28]. PA inhibits myosin phosphatase by binding with high affinity to a PA binding site on PP-1α and PP-1β, isoforms of PP-1 which can act as the catalytic subunit of myosin phosphatase (MP) [29]. This binding site is also found in PP-1c and PP-1δ [16,18]. PLD activity is increased in non-adherent cells and PA derived from PLD2 was shown to recruit phosphatidylinositol-4-phosphate 5-kinase Iγ*b* to membranes and thereby to activate β1 and β2 cell surface integrins to promote cell adhesion [30]. In response to PLD2 stimulation, PA accumulates and also recruits MP to the plasma membrane, keeping it in an inactive form [29]. After cells have attached, PLD2 is downregulated, and PA levels decrease, allowing MP to become active at the plasma membrane where it dephosphorylates myosin and thereby promotes the depolymerisation of actomyosin microtubules during cell spreading dynamics [29]. There is evidence that PA binds to the myosin targeting subunit (MBS) of myosin [29].

PA on the membrane binds specifically to the serine/threonine kinase mTOR. The crystal structure of the FKBP-rapamycin-binding (FRB) domain of mTOR co-crystallized with PA revealed that a stretch of five amino acids contribute to anchoring PA to the binding site in the FRB domain which is also lined with hydrophobic residues but only an Arg residue was absolutely essential for the interaction [31]. Mutagenesis studies confirmed that the Arg residue interacts with PA likely via hydrogen bonding between the phosphate group on PA and the side chain hydrogen of Arg [32]. PA is required for the assembly of

both mTORC1 and mTOR complex 2 (mTORC2) [33]. Cell proliferation and survival signalling are mediated by specific interactions of PA with mTOR and PLD activity was also required for PA-mediated mitogenic signalling by mTOR in a variety of cell types [34,35].

PA is also involved in regulating vesicle trafficking and endocytosis, where PA regulates membrane curvature [29]. PA interacts with cAMP phosphodiesterase 4A1 (PDE4A1) in a Ca^{2+}-dependent manner and this interaction regulates stress fibre formation [36–38].

1.2.3. Roles of diacylglycerol as a bioactive signalling lipid

Conversion of PA to DAG in the PLD pathway is a critical step because it can terminate the signalling effects of PA. DAG is a second messenger neutral lipid that can be present in the cell in many fatty acyl species including mono-unsaturated, di-unsaturated, polyunsaturated or saturated [39]. The physiologically relevant form of DAG is *sn* -1,2,-diacylglycerol. Basal levels of DAG in the cell are very low and account for 0.1% to 1% of lipids in membranes [40,41]. DAG derived from PA is reported to stimulate the classical and novel PKC isoforms [4]. Unlike the classical PKCs, the novel PKCs (PKCδ, ε, θ, and η) require recruitment by DAG but lack a requirement for Ca^{2+}-binding for activation [42]. The classical and novel PKCs cannot bind membranes with high affinity until

their C1 domain becomes tethered to DAG in the membrane [43]. However, some evidence suggests that DAG species produced from PA by the PLD pathway activate PKCs to a much lesser extent than DAG species produced from PIP_2 by PLC [44]. DAG mediates inflammatory signalling by activating PKCε. DAG also recruits the Ras guanyl nucleotide-releasing proteins (RasGRPs) to the membrane. Not only does DAG bind directly to RasGRP3, but DAG-dependent PKC isoforms are required to phosphorylate RasGRP3. DAG activates chimaerins, Munc13, and PKD by promoting their recruitment to membranes [4]. DAG is also synthesized at T-cell junctions [45]. Conversion of DAG to PA and PA-enrichment on membranes is also required for T-cell receptor (TCR) signalling.

1.2.4. Regulation of diacylglycerol signalling by diacylglycerol kinases

Diacylglycerol kinase (DGK) activity is the main activity responsible for metabolizing DAG that accumulates in signalling [4]. There are 10 currently identified DGK isozymes in mammalian cells that are localized to the cytoplasm, nucleus, actin stress fibers, Golgi, and ER [46–48]. In some cell types, some DGK isoforms promote cell survival and cancer cell growth and the loss of some DGK enzymes is associated with degeneration in the brain and apoptosis. It is noteworthy that PA produced via DGKα is required for Rab-coupling protein (RCP) to activate invasion in cancer cells [49]. Furthermore, DGKζ knockout mice showed decreased levels of PA in the hippocampus which was associated with loss of dendritic spines [50,51].

However, DGKζ is a negative regulator of signalling by the T cell receptor (TCR) [52]. Overexpression of DGKζ resulted in inhibition of TCR-dependent signalling including downregulation of nuclear factor k-B (NF-κB) activation and interleukin-2 (IL-2) levels decreased in conjunction with a lack of proliferation of peripheral T cells [52]. DGKζ knockout mice exhibited decreased PA levels and increased proliferation of T cells accompanied by elevated levels of IL-2, which is associated with the immune response to viral infection or tumorigenesis [52]. DGKζ therefore acts as an inhibitor of the T-cell response to infection [52]. DAG also activates interleukin-1 (IL-1) production in neutrophils [52]. PA can stimulate the exocytosis of neutrophils in a manner that is dependent upon DGK activity [53]. In contrast, apoptosis was activated in T lymphocytes in which DGKα was inhibited and this occurred by a Fas ligand-dependent mechanism in which Fas ligand was produced by lethal exosomes [42,46].

However, understanding the functions of DAG signalling is complicated by the large number of DGK isozymes [4]. Knockout of DGK isoforms is also associated with increased recruitment of downstream effector proteins such as chimaerins, PKC isozymes and RasGRP to membranes [46,47]. Similarly to PKCs, DAG kinases are recruited to membranes via their C1 domain [47]. However, DGKζ binds directly to RasGRP and inhibits TCR-dependent Ras activation [52]. DGKζ knockout mice exhibit increased ERK1/2 activation in the T cells and this effect is likely due in part to increased Ras activation [52]. DGKι knockout mice show increased activation of Rap1 GTPase, which in turn

activates the Ras/ERK pathway [46].

1.2.5. Introduction to phospholipase D enzymes

PLD enzymes catalyze hydrolysis of the phosphodiester bond of phosphatidylcholine (PC) to form free choline and PA [54]. PLD activity produces 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid as the predominant lipid species in mammalian cells [55]. The PLD enzymes also show less efficient catalytic activity towards phosphatidylethanolamine (PE) [55]. There are two well-characterized mammalian isoforms of PLD, PLD1 and PLD2. PLD1 and PLD2 are peripheral membrane proteins and show about 50% sequence identity [56]. Water acts as a nucleophile in the PLD-catalyzed transphosphatidylation reaction [54]. The domain structure of PLD1 and PLD2 contains four catalytic domains, each with an HKD motif in the form H(X)K(X_4)D (Fig.1.3) and three phosphoinositide binding domains [57,58] and (Fig. 1.2). PLD1 contains an additional loop segment flanking the polybasic motif and one of the catalytic domains. The loop unique to PLD1 is hypothesized to act as a negative regulator of activity since deletion of the loop results in elevated activity [35]. The pleckstrin homology (PH) and Phox (PX) domains found at the N-terminus have a regulatory membrane-targeting function and bind phosphoinositides [29]. The PLD isoforms also contain a polybasic motif composed of several positively charged residues forming a sequence that is able to bind to PIP_2 [59,60]. PLD1 is palmitoylated at Cys240 and Cys241 residues located in the PH domain (Fig.

1.2) and this modification is required for membrane localization and endocytic trafficking after stimulation with phorbol myristate acetate (PMA) [61,62]. Besides fatty acylation, PLD1 is also post-translationally modified by monoubiquitination [63].

PLD also catalyzes a transesterification reaction in which a primary alcohol, when supplied to the enzyme, preferentially acts as the nucleophilic acceptor, resulting in formation of phosphatidyl alcohols [64]. This transphosphatidylation reaction catalyzed by PLD is used to measure PLD activity based on the production of phosphatidylacohols which are not otherwise found in cell membranes. Many studies of PLD activity have utilized primary alcohols such as ethanol or 1-butanol, which compete with water as a nucleophile to prevent the formation of PA [54]. Recently, PLD isoform-specific small molecule chemical inhibitors were developed as a means to attenuate PLD activity [65].

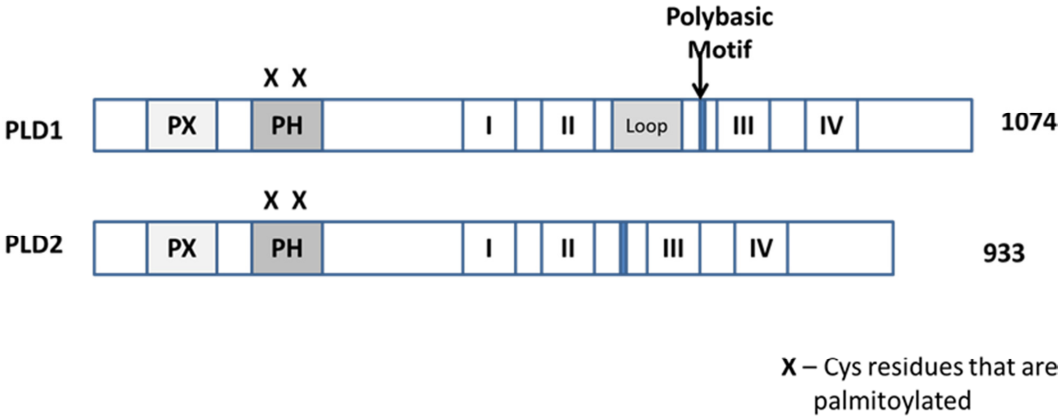


Figure 1.2. The domain structure of mammalian PLD1 and PLD2. The loop region is only found in PLD1. The cysteine residues that can be palmitoylated are indicated by X. The positions of HKD motifs are indicated by the Roman numerals I,II,III and IV. Abbreviations are as follows: Phox (PX), Pleckstrin homology (PH).

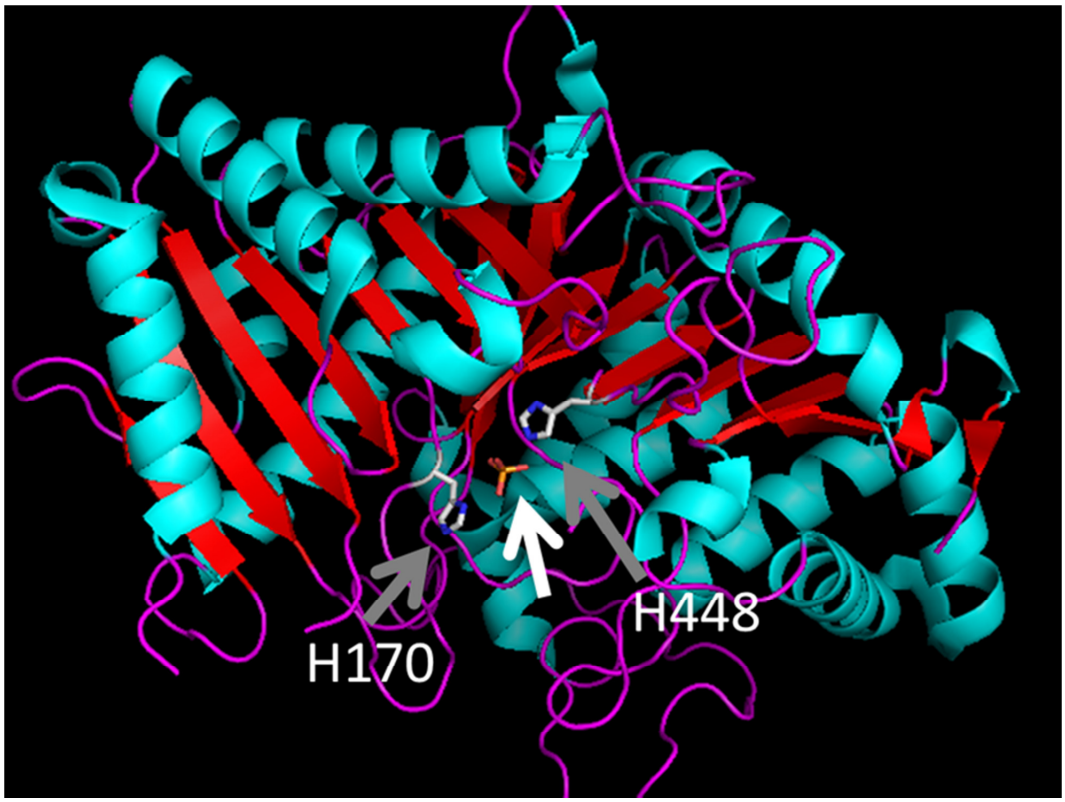


Figure 1.3. Crystal structure of PLD from *Streptomyces sp.* This structure is composed of two domains formed from one polypeptide chain. The structure is at 1.4 Å resolution and is complexed with a phosphate group (indicated by the white arrow) in the active site. The active site is buried inside the protein. The structure is colored according to secondary structure. There is evidence that H170 and H448 (indicated by the grey arrows) are the histidine residues that act as proton donor and nucleophile, respectively in the catalytic mechanism. The flexible loops near the active site are believed to contain residues that contact the membrane during catalysis. Image prepared with PyMOL and modified from Leiros *et al* [66] and PDB Accession 1F0I.

1.2.6. Functions and regulation of phospholipase D1 enzymes

PLD1 activity is regulated intracellularly by the GTPases ADP ribosylation factor (Arf) and Ral, protein kinase Cs (PKCs) and the family of Rho GTPases (Fig. 1.4) including RhoA, Rac1, and Cdc42 [67]. Activation of PKCα by endothelin-1 and other protein ligands including epidermal growth factor (EGF),

platelet derived growth factor (PDGF), thrombin and insulin, causes PKCα to phosphorylate and activate PLD1 [56]. PLD1 can also be activated by PKCα and PKCβ *in vitro* by a mechanism that does not involve phosphorylation [56]. PKCs can activate PLD1 by binding to interaction sites at the N- and C-terminus of PLD1 [68]. PLD1 is also activated by the binding of PIP_2 at the PH domain and by Ca^{2+} [64]. PLD1 is activated in response to stimulation of cells by extracellular signalling molecules such as hormones, the PKC activator phorbol myristate acetate (PMA), reactive oxygen species, neurotransmitters and GPCR agonists including LPA [29,64,69]. Stimulation of human lung adenocarcinoma cells with bradykinin or S1P led to the phosphorylation and activation of PLD1 by PKCδ [70]. PLD1 is also phosphorylated at a tyrosine residue, but this modification did not affect the activity [71].

PLD1 is reported to translocate to plasma membranes and to be distributed within sorting/recycling endosomes, lysosomes, and early and late endosomes, with conflicting reports as to whether or not PLD1 localizes to the Golgi [55,72].

1.2.7. Functions and regulation of phospholipase D2 enzymes

PLD2 is activated intracellularly by Ral, PIP_2 , PKCδ and PKCε [29,73,74]. PLD2 is inhibited by actin, α-actinin, synaptojanin [29]. Evidence is also emerging that promotion by PLD2 of cell binding to integrins and localization of PLD2 to lamellipodia is dependent on direct phosphorylation of PLD2 at Thr566

by PKCδ [75] but the activation of PLD2 by PKC remains controversial [29]. It also remains controversial whether ARF small GTPases can activate PLD2 [67].

PLD2 translocates primarily to the plasma membrane in response to treatment of cells with PLD agonists, LPA, and agonists of GPCRs [13,76]. PLD2 is also found in vesicles, in the cytosol, and in β-actin compartments and the Golgi apparatus [77,78]. PLD2 can also be activated by the EGFR as will be discussed below.

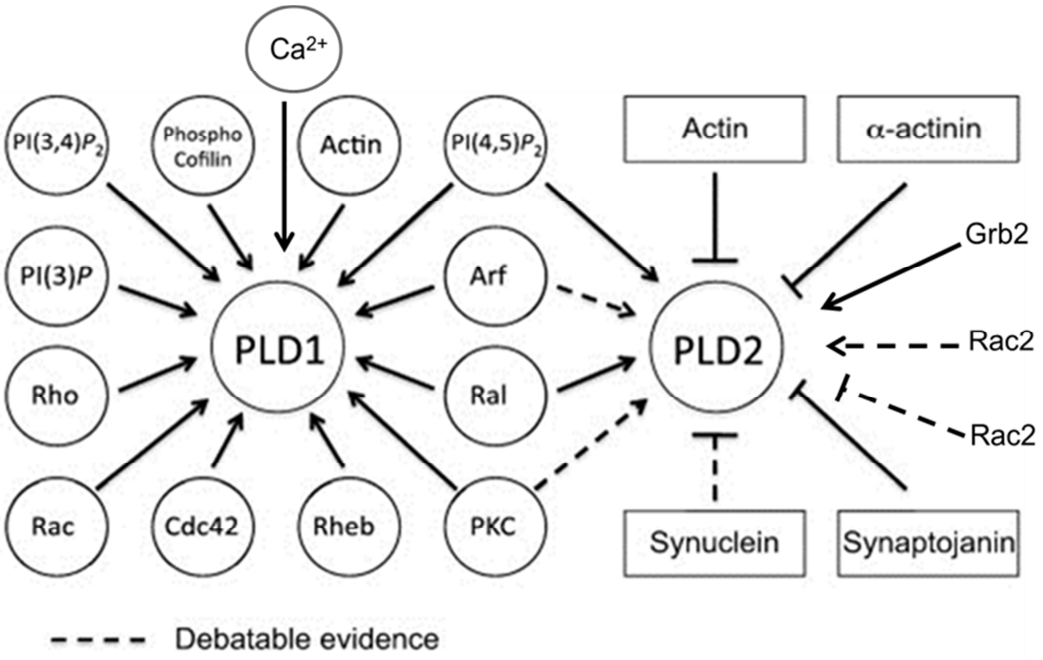


Figure 1.4. Regulators of mammalian PLD1 and PLD2 activity within the cell. Adapted from Rudge *et al.*,[29]. Abbreviations are as follows: phosphatidyl inositol triphosphate (PI(3)P), phosphatidyl inositol-3,4-bisphosphate ($PI(3,4)P_2$), phosphatidyl inositol-4,5-bisphosphate ($PI(4,5)P_2$), protein kinase C (PKC). Evidence is emerging that Rac2 is able to activate and inhibit PLD2 activity in leukocytes [79]. Rac2 activates PLD2 to promote migration in phagocytes and inhibits PLD2 activity during the late inflammatory response after migration is completed [79].

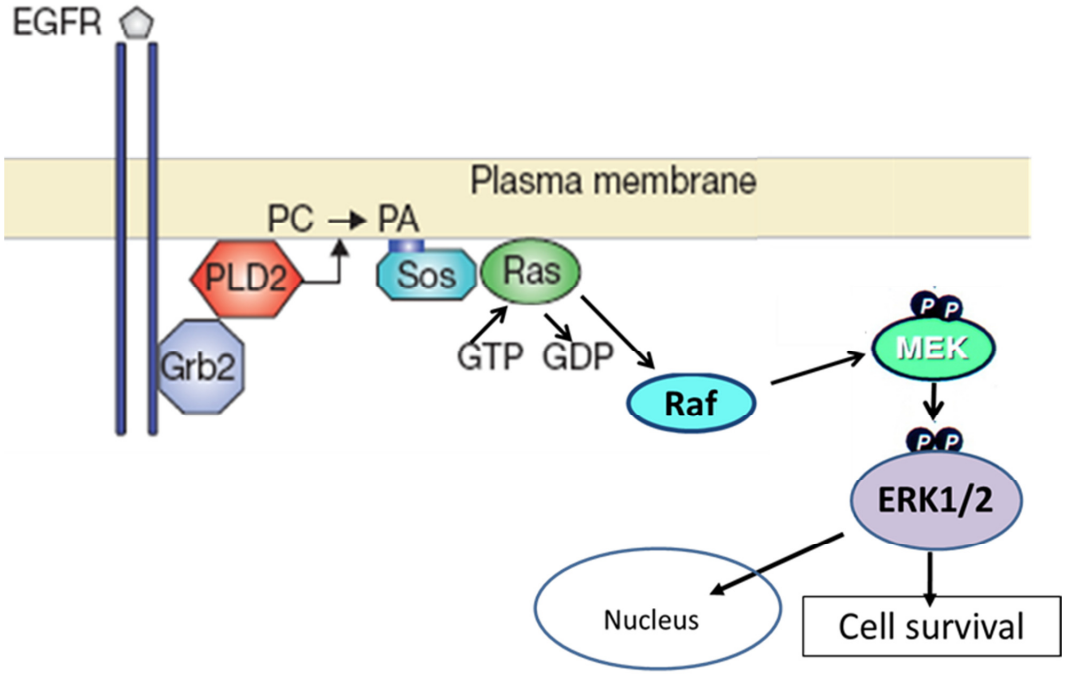


Figure 1.5. PA acts as a signaling lipid to activate ERK1/2 and promote cell survival signaling. Abbreviations are as follows: Epidermal growth factor receptor (EGFR), Phospholipase D2 (PLD2), phosphatidylcholine (PC), phosphatidic acid (PA), mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase 1/2 (ERK1/2). Adapted from Hancock *et al.*,[80].

1.2.8. Interactions of phospholipase D2 and epidermal growth factor receptor signaling

In the MCF-7 breast cancer cell line, the EGFR phosphorylates PLD2 at Y296, an inhibitory phosphorylation site which downregulates PLD2 activity. The EGF receptor (EGFR) can be transactivated by GPCRs including the LPA and S1P receptors [81,82]. Transactivation of the EGFR by LPA in human bronchial epithelial cells was dependent upon PLD2 activity [83]. In Cos7 cells treated with

EGF, PLD2 activity was required for Sos to be recruited to the membrane in order for Sos to activate Ras [13,84–86]. Knockdown of PLD2 inhibited the activation of Ras by EGF [13]. EGF treatment is also reported to activate PLD2 in a number of cell lines including HEK 293, A431, and in Swiss 3T3 cells [87,88]. It has been reported that activation and phosphorylation of PLD2 by the EGFR is PKCα-dependent in Cos7 cells. PA is also required for internalization of the EGFR [89].

The EGF receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases [90]. The EGFR is a single polypeptide glycoprotein that contains an N-terminal ectodomain located on the extracellular side of the plasma membrane [90]. The ligand binding site is located on the N-terminal domain and the tyrosine kinase domain is cytoplasmic and attached to the C-terminal tail [88]. The EGFR is activated when a soluble ligand binds to the receptor ectodomain which allows a conformational change causing the dimerization loop to become accessible, which promotes homo-dimerization and activation [91]. Thus activated, the tyrosine kinase domain phosphorylates multiple tyrosine residues on the C-terminus [92]. The phosphotyrosines are binding sites for adaptor proteins and proteins which regulate EGFR activity including the SHP phosphatases [93]. Grb2, AP2, and proteins in the SH2 domain-containing family are among the phosphotyrosine binding protein adaptors recruited to the EGFR that activate the MAPK pathway [85]. PI3K is also recruited in response to EGFR phosphorylation and promotes cell survival [90]. The activation of the EGFR leads to a signalling network where multiple biological outcomes are possible.

EGFR autophosphorylation and recruitment of GRB2 is also known to recruit casitas B-lineage lymphoma (CBL), an E3 ubiquitin ligase that promotes the internalization of the EGFR and its recycling via endosomal pathways [94]. In HEK 293 cells, it was found that EGF treatment strongly stimulated PLD1 activity but PLD2 showed less activation in response to treatment, although this result remains controversial [95]. PLD1 association with the EGFR was stimulated by EGF whereas PLD2 formed a binding interaction with the EGFR that was independent of EGF binding [95]. Dimerization of the EGFR also promotes its ubiquitination [94]. Ubiquitinylated EGFR could recruit proteins required for endocytosis which contain motifs that recognize ubiquitin such as Eps15 and Epsin1, and this is required for the EFGR to be targeted to lysosomes for degradation [90].

EGF is a mitogenic protein that can activate signalling in many cell types [92]. Evidence suggests EGF treatment of cells promotes the association of PLCγ1 with the EGFR and PLC is phosphorylated [90]. The activation of PLC by the EGFR does not cause Sos translocation to the plasma membrane or Ras activation [13]. The Rac-GAP β2-chimaerin is recruited to the membrane by the EGFR and promotes DAG production via PLCγ activity [92]. It was shown that DGKδ promotes the de-ubiquitination of the EGFR via PKCα and thereby attenuates its degradation [96]. The EGFR can also be activated in a ligand-independent manner, which frequently occurs in cells with mutations in EGFR and is associated with tumorigenesis in the brain [92]. Ligand-independent activation of the EGFR also can be mediated by E-cadherin-promoted cell-cell

adhesion [96] and by oxidative stress [92,97]. Ligand-independent activation of the EGFR is a feature of a number of cancers including ovarian, lung, and breast cancers [92].

1.2.9. The importance of phospholipase D signalling in cancer

PLD plays an important role in promoting cancer progression. Both PLD expression and activity is upregulated in many different cancers including gastric, colon and breast cancers [98–101]. PLD activity was required for secretion of matrix metalloproteinase (MMP) enzymes, including MMP-9 in colorectal cancers and MMP-2 in gliomas [102,103]. Much evidence from the recent literature shows that PLD is responsible for important signalling events in breast cancer cells. PLD1 is reported to be overexpressed in many breast cancers [104]. Elevated PLD activity allowed MDA MB 231 breast cancer cells to survive the stress of serum deprivation and increased the invasiveness in matrigel assays [105]. Elevated PLD activity upon stimulation with PMA was observed in doxorubicin-resistant MCF-7 cells [106]. A positive correlation between doxorubicin resistance and stimulated PLD activity was established. Endothelin-1 activates PLD via the endothelin-1A (ET1A) and endothelin-1B (ET1B) receptors, which are GPCRs [107]. Secretion of endothelin-1 by breast tumors was shown to correlate with invasive phenotypes. Low levels of endothelin-1 were secreted by the non-tumorigenic mammary cell line hTERT-HME1 and by the breast cancer cell line MCF-7 which shows low metastatic potential [108]. In contrast, the invasive cell line MDA MB 231 secreted high levels of endothelin-1.

Endothelin-1 activated signalling through a pertussis-toxin regulated pathway via the MAPK pathway. PLD activity also contributes to rapamycin resistance in MCF-7 cells [109]. Moreover, EL4 lymophoma cells exhibit increased activation of PLD2 and PLD2-dependent activation of focal adhesion kinase and phosphorylation of Akt and this correlated with increased invasiveness [110].

Furthermore, in peripheral blood mononuclear cells, stimulation of PLD activity with the n-3 fatty acid docosahexaenoic acid (DHA) followed by treatment with the mitogenic lectin concanavalin A resulted in a decreased mass of PA and a corresponding increase in labelled DAG [111]. This evidence showed that activation of PLD may result in increased DAG levels rather than PA accumulation. Subsequent work associated DHA treatment with decreased breast, colon, and lung cancer growth through the ability of DHA to alter EGFR-dependent signalling [112].

PLD is beginning to emerge as a potential target in cancer therapy, but there are still many unanswered questions about the regulation of the PLD pathway and regulation of PLD signalling by bioactive lipids [79]. In particular, LPA can promote breast cancer cell survival and signalling through activation of PLD [5]. Of note, PLD1 knockout mice display a decreased ability to carry out macroautophagy, a process in which the cell recycles nutrients and that is often employed to support cancer growth [113,114]. Furthermore, halopemide a psychotropic drug in use since 1970 and recently demonstrated to be a dual PLD1/2 inhibitor can be given safely in humans based on five clinical trials [115]. This evidence points to the possibility of safely targeting PLD in cancer patients

[116]. Thus, it is important to understand how to attenuate PLD signalling in cancers and in particular breast cancer.

1.2.10. Role of phospholipase D in immune signaling

PLD is activated by fMLP, a chemotactic peptide agonist of its GPCR [56]. PLD1 is also responsible for activation of the Fcγ immune receptor in immune signalling in neutrophils [117]. In RAW 264.7 macrophages, LPS treatment promoted a transient increased in DAG levels and this increase was inhibited by butan-1-ol, which blocks PA formation by PLD [118]. PLD promotes the recruitment of talin in human neutrophils and this process requires PLD-derived PA, which promotes accumulation of PIP_2. Chemokine stimulation in human T lymphocytes caused PA generated by PLD1 to promote a fast activation of LFA-1, a β2 integrin. Interleukin-8 sequentially activates PLD2 mRNA expression followed by mTOR and S6-Kinase expression [119]. Studies in PLD2 knockout mice show that PLD1 rather than PLD2 regulates PMA-induced chemotaxis and reactive oxygen species production in neutrophils [117]. DAG can also activate protein kinase D (PKD) and this is required for cytoskeletal remodelling, proliferation, and immune cell signalling. DOCK2, a GEF for Rac, binds PA through polybasic regions to mediate neutrophil chemotaxis [34]. This recruitment was PLD2-dependent and stimulated by EGF [13]. Despite the

importance of PLD in immune signalling and in the progression of cancer, little is known about the enzymes which regulate the metabolism of PA.

1.2.11. The phosphatidic acid phosphatase which degrades phosphatidic acid in the phospholipase D pathway

PA constitutes only about 1% of total cellular lipids under basal conditions [120,121]. Because of its role as a second messenger, the intracellular level of PA is tightly regulated through synthesis and turnover [3]. PA levels rise rapidly upon stimulation of the PLD pathway. The chemical steps of the PLD pathway downstream of PLD activity that control the balance in the formation of the two bioactive lipids, PA and DG have not yet been firmly determined [122]. In order to understand the regulation of signalling by PA and DG downstream of PLD signalling, it is necessary to elucidate which PA phosphatase is involved. So far, the enzymes that act downstream of PLD to degrade the PA formed by PLD have not yet been conclusively identified [123]. There are two families of PAPs which can potentially dephosphorylate PA to DAG and these enzymes are the lipins and the LPPs.

1.3. The Lipid Phosphate Phosphatases

1.3.1. Introduction to the lipid phosphate phosphatases

The LPPs consist of three isoforms, LPP1, LPP2, and LPP3, and a splice variant LPP1a [24]. The LPPs dephosphorylate a variety of phosphate esters including PA, S1P, LPA, diacylglycerol pyrophosphate, N-oleoyl ethanolamine phosphatidic acid, ceramide-1-phosphate (C1P) and diacylglycerol pyrophosphate [124,125]. Bioactive lipids such as circulating LPA and S1P can activate cell surface receptors whereas intracellular LPA and S1P can act as lipid second messengers [126].

1.3.2. Roles of the lipid phosphate phosphatases in extracellular signaling

LPPs contain six transmembrane domains which are inserted in the plasma membrane with active sites facing the extracellular side of the plasma membrane [22,127,128]. Both LPP2 and LPP3 localize to detergent-resistant lipid rafts [60]. The LPPs are ecto-enzymes which are likely to participate in signal transduction by reducing the levels of extracellular bioactive lipid phosphate esters that can activate cell surface receptors [24,129]. LPA and other polar lipid substrates of the LPPs including S1P and PA cannot be taken up by cells rapidly. However, their dephosphorylated forms can rapidly enter the cell and the ecto-activity of the LPPs thus control the levels of lipid phosphate phosphatases versus their cell-permeable dephosphorylated products. LPPs can

inhibit the signalling pathways initiated by lipid phosphate signalling [24]. Overexpression of LPP1 caused increased dephosphorylation of extracellular lipid phosphate esters PA, LPA, and C1P [124]. The LPPs hydrolyze extracellular LPA to monoacylglycerol (MAG) by their ecto-activities [129]. MAG formation terminates the extracellular signalling effects of LPA [124]. Extracellular MAG can be internalized by cells and phosphorylated to LPA by acylglycerol kinases.

LPPs can thus control the level of LPA in circulation which is available to accumulate in the microenvironment of developing tumors. Elevated LPA concentrations up to 10 µM are observed in cancer patients compared to concentrations ranging from 100 nM up to 2 µM in normal circulation [5]. In LPP1 hypomorph transgenic mice, which have LPP1 expression that is reduced by about 90% in most tissues, the half life of LPA in the circulation was increased [124,130]. LPP activity could play a substantial role in preventing cancer progression because extracellular LPA can activate PLD1 and PLD2 [131]. In ovarian cancers and ovarian cancer cell lines, the ecto-activities of LPP1 and LPP3 are low and this was linked to cancer growth and metastasis. Gonadotropin releasing hormone increases LPP expression in ovarian cancer cells and it also prevents proliferation in ovarian cancers. LPP1 expression is decreased in ovarian tumors and in ovarian cancer cell lines, which could result in elevated levels of extracellular LPA and increased extracellular signalling.

S1P is a sphingolipid analogue of LPA. Extracellular S1P forms a component of plasma and can be found at high nanomolar concentrations in plasma in association with lipoproteins, albumin or erythrocytes [23]. The

concentration of S1P in the circulation ranges from 100 nM to 1 μM and S1P is found within the cell only at low nanomolar concentrations [41]. S1P is reported to play multiple roles in cell differentiation and development by signalling through GPCRs [132]. There are currently five S1P receptors that have been identified. S1P also activates p42/p44 MAPK. S1P promotes cell division, angiogenesis, and increased S1P production is associated with increased tumor growth [19]. LPP1a and LPP3 hydrolyzed the S1P analogue FTY720 phosphate preventing FTY720 phosphate-dependent activation of S1P receptors [133,134]. LPPs can hydrolyze S1P to sphingosine by their ecto-activities, and this prevents activation of S1P receptors and pro-survival signalling by S1P [22]. The sphingosine that is formed is taken up by cells and converted back to S1P [25]. Alternatively, sphingosine can enter the cell and be converted to ceramide by ceramide synthase [41]. Ceramides and sphingosine promote cell cycle arrest and apoptosis in response to cell stress, compared to S1P which contributes to cell survival [135,136].

1.3.3. Roles of lipid phosphate phosphatases in intracellular signaling

LPPs can also be associated with intracellular membranes including membranes of the Golgi, endoplasmic reticulum, endosomes and lysosomes where they are involved in intracellular signalling [127,137]. Intracellular functions of LPPs could regulate chemoresistance and cell migration [24]. Intracellular LPA

can be formed from PA by the activity of Ca^{2+}-independent PLA_2 ($iPLA_2$) and Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) enzymes. Intracellular LPA can also be converted to monoacylglycerol (MAG) by the LPPs. Intracellular LPA can activate peroxisome proliferator activated receptor γ (PPARγ) receptors and LPA receptors in the nucleus [131].

Evidence that LPPs control intracellular signalling comes from the demonstration that thrombin activates the phosphorylation of ERK1/2 and this effect was abrogated by overexpression of LPP1, LPP2, and LPP1a [138,139]. Thrombin is an agonist of the Gi/o-coupled receptor and thrombin cannot be hydrolyzed by LPPs. This indicates that inhibition of ERK1/2 activation cannot be due to the ecto-activity of the LPPs but that LPPs are responsible for regulating intracellular signalling [139]. Moreover, the ratio of PA to DAG decreased when LPP1 or LPP2 was overexpressed, providing evidence that LPPs could inhibit ERK activation by preventing PA accumulation [139]. Similarly, overexpression of LPP1 inhibited PDGF-dependent fibroblast migration and it was suggested that the inhibition of migration resulted from an accumulation of DAG [129]. DAG concentrations in LPP1-overexpressing cells were chronically elevated, causing the cells to decrease PKC expression, which would inhibit cell migration [140]. LPP2 also regulated intracellular levels of PA whereas LPP3 regulated intracellular levels of S1P [22]. Pilquil *et al* showed that under conditions in which the degradation of extracellular LPA did not exceed 10% of added LPA, overexpression of LPP1 inhibited LPA-dependent stimulation of PLD activity, ERK1/2 activation, and migration of fibroblasts [131]. PLD2 activity was required

for LPA-stimulated migration but not for PDGF-stimulated migration [131]. Depletion of LPP1 activity promoted LPA-stimulated cell migration but not PDGF-stimulated migration [131]. These results supported a role for LPP1 in intracellular signalling that is independent from its ecto-activity [131].

LPPs could also regulate cancer progression by effects of intracellular signalling. The intracellular ratio of PA/DAG was decreased when LPP1 or LPP2 were overexpressed [139]. LPP2 overexpression is detected in many cancer cell lines and it may promote cell proliferation [141]. Consistently, p53 exerts an inhibitory effect on the transcription of the PPAP2C gene encoding LPP2 and p53 has been shown to inhibit other genes involved in regulation of the cell cycle such as cdc25A, cdc25c, cdc20 and chk1 [141]. Depletion of LPP2 inhibited the anchorage-dependent proliferation of cancer cells *in vitro* [141]. LPP2 knockdown also delayed the progression into S-phase of the cell cycle in Rat2 fibroblasts, thus promoting the gradual onset of senescence accompanied by eventual arrest in G2/M transition [142]. LPP2 overexpression led to early entry of cells into S-phase which was associated with increased expression of Cyclin A at a premature stage [142]. The catalytic activity of LPP2 was required for premature entry into S-phase and overexpression of catalytically inactive LPP2 had no effect on entry of cells into S-phase [142]. LPP2 expression is upregulated in transformed fibroblasts and in the MCF-7 breast cancer cell line and in the human sarcoma cell lines MG63, U2OS, and SK-LMS1 [141]. Microarray studies showed that LPP2 expression is increased in transformed human adult mesenchymal stem cells as compared to the non-transformed

control cells [141]. In contrast to the knockdown of LPP1 or LPP3 which does not regulate entry into the cell cycle, knockdown of LPP2 activity decreases cancer cell growth and LPP2 is considered a target in cancer therapy.

1.3.4. Evidence for and against a role of lipid phosphate phosphatases in phospholipase D-mediated signaling

The current literature on PLD signal transduction suggests that the LPPs are the main phosphatidic acid phosphatase (PAP) enzymes that are downstream of PLD activity and metabolize PA [60,123]. Overexpression of LPP2 but not LPP1 caused DAG levels to increase in response to PMA stimulation compared to unstimulated controls [60]. Furthermore, overexpression of PLD1 or treatment of cells with PMA caused LPP3 to be localized to the perinuclear region where it was colocalized with SK-1 [22]. PLD2 and LPP3 were found to be colocalized at caveolae [60]. Not only did treatment of cells with LPA cause a decrease in activation of ERK1/2 in LPP2- or LPP3-overexpressing cells compared to controls, but treatment of cells with 2S-OMPT, a thio-LPA analogue that was resistant to the phosphatase activity of LPP also caused a decrease in ERK activation in response to LPP2 or LPP3 overexpression. This suggested that the role of LPPs in ERK activation did not involve hydrolysis of LPA and that instead the LPPs were playing an intracellular role in ERK

activation. Overexpression of LPPs caused inhibition of ERK1/2 activation by their intracellular activity rather than by their ecto-activity.

However, a role for the LPPs in metabolizing PLD-derived PA is not consistent with the membrane topology of LPP enzymes. LPPs are integral membrane proteins in which the catalytic residues which constitute the active site are predicted to be on the extracellular surface of the plasma membrane or lumenal surface of internal membranes [143,144]. PA, which is formed by PLD1/2 is predicted to remain on the cytosolic leaflet of membranes. Studies using fluorescent analogues of PA demonstrate that PA is converted to DAG in order to traverse the membrane [145,146]. It has been demonstrated that the rate of diffusion of PA from the inner leaflet of the lipid bilayer to the outer leaflet is slow, which suggests that transport of PA across the bilayer is not catalyzed by translocases to a significant extent [147]. Thus, it remains unclear how the LPPs could access PLD-derived PA. Recent work showed that LPP1 overexpression in Rat2 fibroblasts or LPP2 and LPP3 overexpression in HEK293 cells inhibits LPA-stimulated PA accumulation [22,131]. However, the role of LPP1 in decreasing PA levels can be explained by the observation that PLD activation by LPA or PDGF is also inhibited in fibroblasts overexpressing catalytically active but not inactive LPP1 [131]. In addition, knockdown of LPP1 with siRNA caused an increase in PLD activity in Rat2 fibroblasts [131]. Furthermore, overexpression of LPP1 also decreased activation of Rho-GTP, ERK and Ca^{2+}-transients [124,131]. Thus decreased PA levels may result from decreased PLD activity rather than an effect of PA hydrolysis by LPPs. Overexpression of LPP1 was also

shown to inhibit the PLD2-dependent migration of fibroblasts [131]. The LPPs may act upstream of PLD activation as negative regulators and the lipins may be the main PAP enzymes downstream of PLD activity that convert PA to DAG [131]. Therefore, the question remains undecided as to whether LPPs are playing a role in the PLD pathway.

LPA promotes intracellular signaling by activating the LPA_{1-8} receptors, which are G-protein-coupled receptors that signal through heterotrimeric G proteins including $G_{i/o}$, G_s, G_q, and $G_{12/13}$ [24,129]. Further evidence for a role of LPPs in intracellular signaling came from studies with wls-31, phosphonate LPA analogue that activated LPA_1 and LPA_2 receptors [148,149]. The ecto-activity of LPPs was unable to hydrolyze this LPA analogue [148]. The phosphonate analogue promoted cell migration in fibroblasts and this effect was blocked by increased expression of LPP1 [131]. These studies utilized high concentrations of LPA which ensured that hydrolysis of extracellular LPA did not affect cell migration promoted by LPA [131]. These experiments showed that increased LPP1 activity decreased the PLD activity and cellular PA levels. Decreased PLD activation attenuated the effect of LPA or the LPA analogue in promoting fibroblast cell migration [131]. The mechanism of increased cell migration in response to LPA activation was dependent upon activation of Rho and ERK1/2.

1.4. Roles of Lipins and the Yeast Phosphatidic Acid Phosphatase (PAH1) Enzyme in Signalling

1.4.1. Introduction to the lipins

The LPPs are not likely to hydrolyze PA derived from PLD activity and the other candidates for a role as PA phosphatase in the PLD pathway are the members of the lipin family. In contrast to the LPPs, the lipins show phosphatase catalytic activity that is specific to PA as a substrate [150]. The lipins are multi-functional enzymes whose canonical roles involve activity as transcriptional coactivators in the nucleus in addition to their role in converting PA to DAG in the glycerolipid synthesis pathway as will be discussed below [151]. There are three mammalian lipin isoforms termed lipin-1, lipin-2, and lipin-3 [151]. The lipin isoforms have about 45% sequence similarity [151]. Lipin-1 consists of three splice variants, generated by alternative mRNA splicing, called lipin-1α, lipin-1β and lipin-1γ. The lipin-1γ variant is the main lipin isoform expressed in brain tissue [152] and the primary sequence contains a short sequence that is not present in the other two splice variants [153]. The gene encoding lipin-1 was discovered by positional cloning to be mutated in fatty liver dystrophy (*fld*) mice. The *fld* mouse contains a null mutation in the *Lpin1* gene [154].

All three lipin isoforms have PAP activity [155]. The PAP activity of lipins is dependent on the presence of divalent cations such as Mg^{2+} whereas the LPPs do not require metal cations for catalysis [123]. PAP activity of the lipins is also inhibited by the Ca^{2+}-salt form of PA. Mg^{2+}-dependent PAP activity exhibits

surface dilution kinetics in Triton-X-100 micelles [156]. Lipins are able to form homo- and hetero- oligomers that are resistant to solubilisation with detergent [157]. However, crystal structures of a lipin protein are not yet available and therefore the understanding of the structure of the active site and mechanism of catalysis of the lipins remains limited.

The PAP activity due to lipins is sensitive to inhibition by the alkylating agent *N*-ethylmaleimide (NEM), whereas LPP activity is not inhibited by NEM [129]. The sensitivity of the phosphatase activity of lipins to NEM suggests that binding of NEM may alter the conformation of the enzyme since cysteine residues do not appear to be involved in catalytic mechanism predicted for phosphatases such as the lipins [158]. Structural analyses based on the sequence of lipins showed that the lipins belong to the haloacid dehalogenase domain (HADD) superfamily of enzymes that include FCP1, an enzyme that dephosphorylates the C-terminal domain of RNA Polymerase II in association with Transcription Factor IIF [159]. Sequence homology studies have predicted that there are four candidate HADD motifs (Fig.1.6) within the C-terminal sequence of lipins [160]. The first motif contains the two catalytic aspartate residues, one of which is a predicted nucleophile [160]. The second motif contains a hydroxyl group from a serine/threoine residue that participates in coordinating the phosphate from the substrate, the third motif contains a lysine or arginine side chain and the fourth motif contains two aspartate residues that are likely in a position to coordinate the Mg^{2+} ion in the active site of phosphatases in the HADD superfamily [158]. The HADD motifs contain the catalytic residues

that are predicted to line the active site of lipins and to specify the reaction mechanism [158].

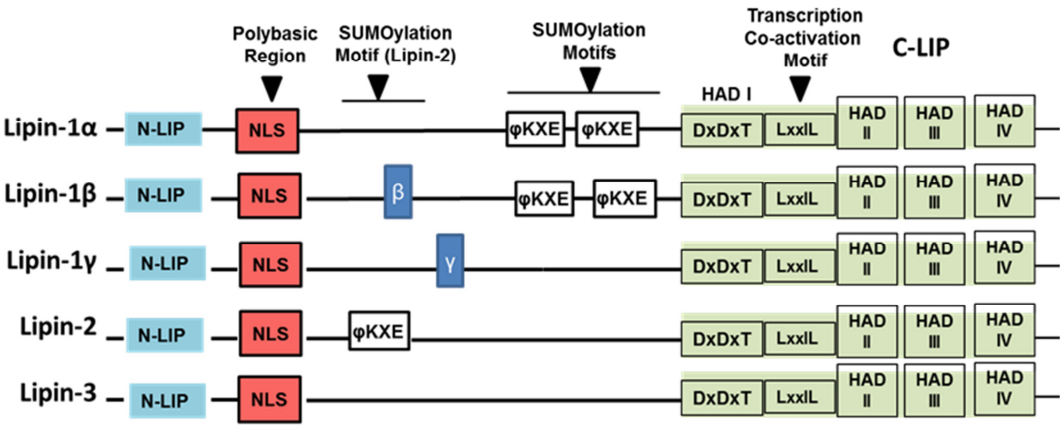


Figure 1.6. Domain structure of mammalian lipin isoforms. The C-LIP domain is highlighted in green. The HAD I domain contains the conserved sequence DxDxT, The conserved sequences are described in detail in the accompanying text. The β and γ symbols represent sequences that are unique to lipin-1β and lipin-1γ, respectively.

1.4.2. The yeast phosphatidic acid phosphatase ortholog of lipin, PAH1, and its roles in signaling

The yeast ortholog of lipin-1, PAH1 encodes a soluble PAP enzyme [150]. Many invertebrates including yeast possess only one ortholog of lipin whereas bony vertebrates possess many lipin orthologs [161]. Studies in yeast have shown that the PAP activity of PAH1 is essential for production of PA, which inhibits transcriptional activation of phospholipid synthesis genes [162]. Moreover, in the budding yeast *S. cerevisae*, PA in the ER membrane acts as a

pH sensor [163]. The Opi1 transcriptional repressor interacts with Ino2p/4p to inhibit activation of genes containing an upstream activating sequence responsive to inositol (UAS^{INO}) that are required for glycerolipid biosynthesis [164]. Elevated PA levels increased the binding of Opi1 to PA and Scs2 on the ER membrane surrounding the nucleus, which prevents translocation of Opi1 to the nucleus and causes increased expression of INO1 and increased inositol production [163].

Mutation of either catalytic aspartate residue to glutamate in PAH1 resulted in a complete lack of PAP activity and the ability of PAH1 to regulate transcription of the INO1 gene which encodes inositol-3-phosphate synthase was also abrogated [165]. The PAH1 deletion mutant, which lacked PAP activity, showed decreased DAG and TAG levels and this effect was mainly seen during the stationary growth phase of yeast [162]. Mutant PAH1 lacking PAP activity also showed increased levels of PA, fatty acids and sterol esters [165]. Moreover, PC levels were substantially decreased whereas PE and PI levels were increased and this occurred mainly during growth phase in yeast [162]. Decreased PC might be caused by decreased availability of DAG required in the CDP-choline pathway for PC synthesis [150]. Elevated PI levels might be caused by increased production of the PI precursor inositol [162]. PAH1 may also be more directly involved in regulating transcription of phospholipid synthesis genes since Pah1p was detected at promoters of UAS^{INO} genes [161]. However, in *S. cerevisiae*, a triple mutation of the yeast lipin homologue PAH1 and of the

yeast LPP homologues LPP and DPP still displayed PAP activity, suggesting that in yeast there may be another PAP enzyme yet to be discovered [150]. The current understanding of PAH1 suggests that its PAP activity regulates PA accumulation and the activation of transcription in phospholipid biosynthesis genes [161].

1.4.3. Role of lipins in glycerolipid synthesis

The lipins are the PAP enzymes in the Kennedy pathway of glycerolipid synthesis [150,166]. The LPPs are unlikely to be involved in glycerolipid synthesis since glycerolipid synthesis occurs on the cytosolic side of ER and mitochondria whereas the catalytic residues of LPPs face the lumenal or extracellular side [128]. The lipins translocate to the endoplasmic reticulum (ER) where they produce DAG that is a precursor for triacylglycerol synthesis and formation of PC and PE [151]. The Kennedy pathway occurs mainly on membranes of the ER of mammalian cells, although some contribution may come from mitochondrial membranes [167]. The Kennedy pathway is the predominant pathway of TAG synthesis in adipocytes and most other tissues. Glycerol-3-phosphate is converted to LPA by glycerol-3-phosphate acyltransferases (GPATs) of which have been identified isoforms GPAT1 to GPAT4, by acylation with a fatty acyl CoA. In the second step, LPA is acylated to PA by LPA acyltransferases (LPAATs) [151]. The lipins convert PA to DAG by hydrolyzing the phosphate group. The next step in the pathway occurs when

DAG is fatty acylated at the *sn*-3 position by diacylgylcerol acyltransferases (DGATs) to form the neutral storage lipid triacylglycerol (TAG) [168]. DAG is also required for the production of major phospholipids including PC and PE that form membranes in the cell [169]. PC accounts for about 40% of lipids in cell membranes [121].

1.4.4. Roles of the Kennedy pathway in signalling

The DAG produced by the Kennedy pathway can activate PKC and the PA, which is the substrate for PAP enzymes, can bind to mTOR [170]. Thus far, it has not been demonstrated which enzymes upstream of PA production are responsible for this accumulation and whether or not it involves activation of the PLD pathway or originates from *de novo* synthesis.

Increased production of DAG through the Kennedy pathway activates PKCε and the phosphorylation of insulin receptor substrate-1 (IRS-1) at Ser307, which prevents IRS-1 activation by tyrosine phosphorylation and the downstream activation of PI3K [170]. Excess fatty acids are seen in *fld* mice and this condition causes increased phosphorylation on tyrosine residues of IRS-1 and promotes insulin resistance, from which a role for lipin-1 in promoting insulin signalling can be inferred [167]. Glycerol 3-phosphate acyltransferase 1 (GPAT1) knockout mice have lower levels of DAG and decreased activation of PKCε [171].

1.4.5. Regulation of lipin translocation to membranes

In contrast to the LPPs, the lipins are soluble PAP enzymes that lack transmembrane domains. The lipins are partitioned between the cytosol, the nucleus and membranes [172]. The lipins are predominantly cytosolic proteins that translocate to membranes where they could hydrolyze the PA produced by PLD activity [173]. In some cell types such as HeLa cells, overexpressed lipin-2 localizes to the cytosol and the ER under basal conditions [173]. The lipins contain a polybasic motif at the N-terminus which also targets the lipins to the nucleus [174]. Translocation of lipins to membranes is regulated in part by electrostatic interactions between the polybasic motif of lipins and negatively charged membrane lipids such as the substrate PA [175,176]. In some interactions with proteins, PA can function as a lipid anchor by directly binding to positively charged sites on signalling proteins with polybasic motifs [174].

Membrane association of soluble PAP activity is inhibited by amphiphilic cations such as chlorpromazine [177]. Chlorpromazine treatment of rat hepatocytes resulted in PA accumulation and decreased the rate of DAG and TAG synthesis by preventing the association of soluble PAP with membranes and by promoting dissociation of PAP activity from the microsomal fraction of membranes [177,178]. It was concluded that chlorpromazine inhibited the translocation of soluble PAP activity by increasing the positive charge on the membrane [177,178].

In contrast, the polyamines spermine and spermidine promoted the translocation of PAP activity to membranes [178,179]. Similarly, the introduction of negative charges due to phosphorylation of lipins might also disrupt electrostatic interactions with negatively charged membrane lipids. PAP activity cannot be recruited to membranes of cells treated with cationic amphiphile drugs because these compounds interact with PA on the membrane and alter its properties, resulting in phospholipidosis and phospholipid accumulation in lysosomes [175,180].

Concentrations of LPA and PA also regulate membrane curvature and budding of vesicles. The significance of translocation of lipins to membranes is further corroborated by the observation that inhibition of PAP activity causes a change in membrane curvature, although it is unclear whether this is due to soluble PAP or LPP activity [181]. PA and DAG are both conical lipids and induce a negative curvature at the neck of budding vesicles [120]. PA also regulates the dynamics of membranes by its cone shape that is unique among all other intracellular negatively charged phospholipids. The cone-shaped PA prevents tight binding of the head group of PA with headgroups of adjacent membrane lipids, thus forming an insertion in the membrane where proteins recruited to the membrane by PA, such as dynamin, could be docked [120,182].

Treatment of cells with epinephrine or the unsaturated fatty acid oleate caused dephosphorylation of lipin and translocation of PAP activity to membranes [183]. The more highly mobile and dephosphorylated forms of lipin-1 translocated to membranes in response to treatment with epinephrine or oleate

[160]. Treatment with epinephrine causes a decrease in phosphorylation of lipin-1 in a manner that does not alter the intrinsic PAP activity of lipin-1 [183]. The mechanism by which epinephrine activates lipin-1 translocation is unknown but it could require termination of the effect of insulin on hyperphosphorylation. Treatment of MDCK cells with epinephrine also caused a rapid elevation in levels of DAG, which supports a role for translocation of soluble PAP [184]. Alternatively, activation of beta receptors by epinephrine could activate adenylate cyclase and PKA and promote increased lipolysis and DAG formation [185].

In adipocytes, activation of insulin signalling causes sequestration of lipin-1. Lipin-1 binds to 14-3-3β and 14-3-3θ proteins in response to insulin treatment and is sequestered in the cytosol [186]. The 14-3-3 proteins bind to Ser residues adjacent to the polybasic motif on lipin-1 [186]. Consistently, treatment of adipose tissue from rats with insulin caused PA levels to increase [187].

Similarly, the phosphorylation status of lipins is regulated by insulin treatment. Insulin promotes phosphorylation of lipin-1 at multiple sites and decreased lipin association with the microsomal fraction [183]. The hypophosphorylation of lipin favors the translocation of lipin in response to fatty acid accumulation. Lipin-1 has PKA recognition sites and epinephrine could activate PKA to phosphorylate lipin-1 on certain residues and could prevent hyperphosphorylation stimulated by insulin [183]. Phosphorylation of lipins regulates their translocation to membranes and lipin-1 has been shown to be phosphorylated on at least 19 serine and threonine residues [183]. The phosphorylation of lipin-1 is regulated by a number of phosphatases, including

Dullard phosphatase [188,189]. Lipin-1 also contains a consensus sequence for glycogen synthase kinase-3 (GSK-3) and a minimal recognition motif for cyclin-dependent kinase 1 (Cdk1) [183]. During cell division in HeLa M cells, the PAP activity was decreased by phosphorylation of lipin-1 or lipin-2, and this occurred in immunoprecipitates isolated with a lipin-1 or a lipin-2 antibody [173], whereas PAP activity was not affected by phosphorylation of lipin in adipocytes [183].

1.4.6. Insulin signalling promotes formation of cyclic phosphatidic acid via phospholipase D2

The PAP activity of lipin is involved in transcriptional coactivation of peroxisome proliferation-activated receptor γ (PPARγ) [190]. This is one example of the requirement for PAP activity in PLD signalling. PLD is stimulated by insulin to produce cyclic PA (CPA), which accumulates in the nucleus. Some lipids can signal by binding to transcription factors such as nuclear steroid receptors [126,191]. In the nucleus, LPA is an agonist of PPARγ receptors [192]. PLD2 has been shown to inhibit the nuclear hormone receptor (PPARγ) in primary macrophages and adipocytes [191]. In addition, work in adipocytes showed that the dual PLD1/PLD2 inhibitor FIPI caused lipin-1 to translocate to the nucleus where it could carry out its transcriptional coactivator function mechanism [174]. The mechanism involved decreased levels of total cellular PA in response to FIPI which promoted increased nuclear localization of lipin-1β

[174]. FIPI also inhibited the production of CPA [191] and PA [193]. Taken together, this suggests that PLD signalling could promote the translocation of lipin-1 to the plasma membrane and inhibit PPARγ activation by preventing the nuclear entry and the transcriptional coactivator activity of lipin-1 [174].

Lysophosphatidylcholine (LPC), preferentially in a polyunsaturated form [194], is a substrate for autotaxin (ATX) and is converted to LPA by the lysophospholipase D activity of ATX in the circulation [195]. Some CPA is also formed by the activity of autotaxin in blood [196]. In addition to its canonical role as a substrate for autotaxin-mediated hydrolysis which produces LPA [24,144], LPC may also be a substrate for PLD2. LPC is an abundant lipid present in plasma at levels up to 200 μM. PLD2 may hydrolyze LPC to CPA in response to stimulation with insulin or phorbol myristate acetate (PMA) [191,196]. CPA may act as a lipid second messenger by translocating to the nucleus and binding to PPARγ, inhibiting the activity of PPARγ by promoting the binding of PPARγ to the corepressor SMRT [191]. Thus PLD2 activity is responsible for formation of both PA and CPA.

The availability of intracellular LPC as a substrate for PLD2 may be dependent on the activity of cytosolic phospholipase A_2 ($cPLA_2$) since $cPLA_{1/2}$ can hydrolyze membrane PC to produce LPC and PLA_2 enzymes are activated by the PLD2 agonists PMA and insulin [197]. CPA is a competitive antagonist of rosiglitazone, a thiazolidinedione (TDZ) drug which binds to PPARγ. Thus PLD2 activation is likely to affect the efficacy of TDZ drug therapy. TZD drugs are used

to treat insulin resistance in patients with type II diabetes and are synthetic agonists of PPARγ.

Rosiglitazone activates the expression of lipin-1 and promotes the activity of PPARγ. Rosiglitazone has been shown to promote increased lipin-1 expression in subcutaneous white adipose tissue and a smaller increase was also detected in lipin-1 expression in visceral fat. The effect of Rosiglitazone is to promote insulin sensitivity in patients with metabolic syndrome and the hypothesized mechanism is through reduction of fatty acid (FA) esterification and triglyceride synthesis in visceral adipocytes or by diversion of FA esterification to subcutaneous white adipose tissue. Notably, the anti-diabetic drugs pioglitazone, a thiozolidone drug, and harmine induce expression of lipin-1 in adipose tissue whereas the antidiabetic drug metformin had no effect on lipin-1 expression [198]. Excess fatty acids in circulation produce insulin resistance. However, deposition of fatty acids in adipocytes increases insulin sensitivity. This redistribution of fat is compatible with increased insulin sensitivity.

The *Lpin2* gene contains a PPAR binding site downstream of the start site of its promoter, and PPARβ/δ appears to bind to this PPAR site and to be required to activate the Lpin2 gene expression response to fasting [161]. At the onset of adipocyte differentiation, lipin-1α expression is upregulated and activates expression of PPARγ. Insulin inhibits the glucocorticoid-induced increase in lipin-1 expression [199]. Lipin-1 can promote the expression of PPARγ at the onset of adipogenesis and this role may require PAP activity [190]. In *fld* MEFs lacking lipin-1 expression, overexpression of lipin-2 or lipin-3 can

induce the upregulation of PPARγ in order to promote adipogenesis [190]. In mature adipocytes, lipin-1 associates with PPARγ$_2$ in a transcription activating complex [200]. The *fld* mice also exhibit insulin resistance which can result from a complete lack of mature adipose tissue. The lack of adipose tissue in *fld* mice is also linked to atherosclerosis [129].

1.4.7. Translocation of lipin-1 to mitochondria in response to mitochondrial phospholipase D activation

The translocation of lipin-1β to fused mitochondria is an example of a direct regulatory role for the PAP activity of lipins in PLD-mediated signalling. PLD signalling occurs at the mitochondrial membrane. A more recently characterized mitochondrial isoform, mitoPLD, is a soluble protein with an N-terminal transmembrane segment that can become anchored in the outer mitochondrial surface [201]. MitoPLD hydrolyzes cardiolipin to PA at the mitochondrial membrane [58]. Overexpression of mitoPLD caused PA accumulation and a corresponding change in mitochondrial morphology from fragmented to aggregated [58]. Overexpression of mitoPLD caused cytosolic lipin-1β to translocate to the mitochondrial surface. A PA biosensor localized to the mitochondrial surface membrane recruited lipin-1 to the mitochondrial membrane in NIH-3T3 cells [201]. The translocation of lipin-1β dephosphorylates the PA at the mitochondria to DAG. As the pool of PA on the mitochondrial membrane is decreased by the PAP activity of lipin, the mitochondria became more fragmented in appearance [201]. The translocation

of lipin-1β also promoted shortening of mitochondrial tubules [201]. Mitochondria in mammalian germ cells contain germline granules consisting of a dense cluster of ribonucleoproteins and other proteins. These germline clusters are often found between aggregated mitochondria and they generate PIWI-interacting RNA (piRNA), a novel class of small RNAs [202]. PA accumulation on the mitochondrial surface of spermatocytes led to increased formation of germline granules in *fld* mice and lipin-1 [58]. Despite significant translocation of lipin-1 to mitochondria when mitoPLD was overexpressed, no evidence was found for the translocation of lipin-1 to regions of the cell where overexpressed tagged-PLD2 was located [201].

1.4.8. Lipin-1 signalling in the peripheral nervous system of *fatty liver dystrophy* mice

Studies in *fld* mice revealed that lipin-1 regulates intracellular signalling events that promote differentiation of Schwann cells during myelin formation [6,203]. Peripheral neuropathy that progressively worsens from birth is observed in *fld* mice and results from demyelination of Schwann cells [204]. Accumulation of PA was observed in the endoneurium of *fld* mice but not in *Lpin1*-expressing controls. PA accumulation is associated with neuropathy in the peripheral nerves of *fld* mice and therefore lipin-1 must be an enzyme that metabolizes PA required for myelination in the peripheral nervous system of mice. The specific PAP activity of lipin-1 accounts for the majority of Mg^{2+}-dependent PAP activity in the

endoneurium of *fld* mice [6]. The endoneurium of *fld* mice showed high levels of PA despite the presence of a high LPP activity [6].

PA accumulation in sciatic nerve in lipin-1 knockout mice is linked to ERK1/2 activation [6]. The expression of the transcription factor Krox-24 was also upregulated [6]. Although overexpression of LPP causes inhibition of ERK1/2, ERK1/2 activation was observed in *fld* Schwann cells despite high LPP activity. This evidence suggests that lipin-1 hydrolyzes the particular pool of PA that acts as an upstream signal to inhibit the MEK-ERK1/2 signalling pathway and it is possible that lipin-1 thereby regulates the activation of S1P signalling within the cell, although this has not yet been demonstrated.

An inhibitor of MEK1/2, PD98059, was able reverse the effects of stimulating the endoneurium with PA *in vitro*. PLD activity has not yet been shown to be essential for the PA accumulation and increased p42/44 MAPK signalling activation observed in *fld* Schwann cells. However, studies with fluorescent PA biosensors used to perform fluorescence resonance energy transfer (FRET) in COS7 cells showed that EGF stimulation caused PA accumulation at the plasma membrane and at lamellipodia [205]. These studies support the hypothesis that PA accumulation can occur downstream of receptor activation and PLD signalling [205]. Consistently, studies with PA biosensors also demonstrated that PA could be detected at contact-free sections of the plasma membrane where Ras activation was increased rather than at cell-cell junctions where Ras signalling was poorly detected [205].

1.4.9. PAP activity of lipin-1 is required for peroxisome proliferation activated receptor γ (PPARγ) activation in adipogenesis

One of the major function of lipins is to co-activate the transcription of genes required for adipogenesis, fatty acid metabolism, and β-oxidation [161,172,206]. All three lipin isoforms contain a nuclear localization sequence (NLS) at the N-terminus (Fig.1.6) and are able to translocate to the nucleus [174]. The primary sequences of lipin-1α, lipin-1β and lipin-2 contain sumoylation motifs and a nuclear receptor interaction motif (Fig.1.6) consisting of the LxxIL consensus sequence (where x represents any amino acid) [207]. Binding of each of the three lipin isoforms in transcription-regulating complexes is likely through a nuclear receptor interaction sequence formed by a LxxIL motif (Fig.1.6) that is rich in hydrophobic residues and is predicted to form an α-helix and this was confirmed by site-directed mutagenesis for lipin-1 and lipin-2.

Both lipin-1 and lipin-2 function as coactivators of transcription with nuclear receptor transcription factors in the PPAR family and peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) [161]. During transcription-promoting complex assembly, it is likely that lipin-1 binds to other transcription factors rather than binding directly to the PPAR promoter since lipin-1 has no DNA binding domains [172]. Adipocyte differentiation is dependent upon expression of PPARγ. PAP activity is also required for adipose tissue development. Consequently, the *fld* mice have greatly reduced functional mature adipose tissue in fat pads and the *fld* pups present a fatty liver and triglyceridemia in the milk-fed period which resolves upon weaning [204].

Inhibition of ERK activation rescued the PA-dependent inhibition of adipocyte differentiation [190]. The lipin-1 deficient mouse is unable to form active adipose tissue despite the expression of lipin-2 and lipin-3. Lipin-2 mRNA levels are significantly higher in human compared to mouse white adipose tissue and this supports the hypothesis that the lipin-2 or lipin-3 can compensate for lack of lipin-1 [155]. The expression of lipins is reciprocally regulated in HeLa cells [173]. Knockdown of lipin-1 caused increased expression of lipin-2 and lipin-3 in HeLa cells [173].

It is becoming evident that some of the functions of lipins as transcription coactivators may be dependent upon the PAP activity of lipins. Treatment of 3T3-L1 adipocytes with PA inhibited PPARγ expression and the MEK inhibitor PD98059 restored PPARγ expression in cells treated with PA [190]. ERK1/2 was activated in adipose tissue of *fld* mice and mRNA expression levels of c-Myc and the nuclear transcription factor Krox-24 were also upregulated [190]. The finding that both ERK1/2- and Krox-24-dependent signalling are disrupted in adipocytes as well as in peripheral nerves in *fld* mice indicates the importance of lipin-1 signalling and argues that these signalling events are not cell-specific but may be of more universal importance. In lipin-1 depleted adipocytes, PA may recruit ERK1/2 to the membrane where ERK1/2 is phosphorylated and the activated ERK1/2 inhibits adipocyte differentiation. PA accumulation inhibits the transcription of PPARγ in adipocytes and PA accumulation in *fld* mice is ascribed to lack of PAP activity. This could occur through ERK1/2 effectors which translocate to the nucleus and prevent transcriptional activation of PPARγ [208].

Indeed, It seems likely that PAP activity of lipins is required to prevent ERK1/2 activation; however this has not been demonstrated directly by phenotype rescue experiments with catalytically inactive lipin mutants [190]. Activation of ERK1/2 and MEK controls the nuclear translocation of the transcription factors Elk-1 and cAMP response element binding protein (CREB). Elk-1 binds to serum response element (SRE) recognition motifs and CREB promotes transcription by binding to CRE motifs on genes [209]. SRE and CRE elements are found in the promoter of the Krox-24 gene. Like skeletal muscle, adipose tissue from lipin-1-deficient mice contains almost no Mg^{2+}-dependent activity, which might explain the PA enrichment and increased MAPK signalling [6,183,186]. Regulation of PLD activity by lipins could provide a mechanism to attenuate these effects of PA signalling.

1.4.10. A role for lipin-1 in signaling by the Mammalian Target of Rapamycin

Recent work showed that PA accumulates in response to oleate treatment in cardiomyocytes from *fld* mice, and increased phosphorylation of mTOR was detected [210]. Accordingly, lipin-1 may be involved in signal transduction as a regulator of mTOR activation and phosphorylation of p70S6-kinase (p70S6-K), because the ability of mTOR to phosphorylate p70S6-K has been shown to be dependent on the binding of mTOR to PA [211,212]. Hearts from *fld* mice exhibited PA accumulation as compared to wild-type mice,

accompanied by increased endoplasmic reticulum stress and increased phosphorylation and activation of p70S6-K and S6 ribosomal protein [210]. P70S6-K phosphorylates S6 ribosomal protein [213]. Rapamycin is an inhibitor of mTORC1 and PA binds to mTOR in a rapamycin-competitive manner [32]. Insulin promotes the phosphorylation of lipin-1 in rat adipocytes and this phosphorylation is inhibited by rapamycin, raising the possibility that lipin-1 phosphorylation requires mTOR kinase activity [214]. Rapamycin also specifically blocked the phosphorylation of human lipin-1β at Ser106 [183]. However, rapamycin does not affect the PAP activity or translocation of lipin-1 to membranes and the specificity of rapamycin in blocking mTORC1 has also been questioned [183]. Conversely, in response to excess nutrient availability, mTOR inhibits insulin signalling by phosphorylating serine residues of IRS [215].

1.4.11. Mutational analysis of lipin-1 reveals a role for phosphatidic acid phosphatase activity of lipin-1 in signaling

The PAP activity of lipins could be required to metabolize PA in the PLD pathway. The lipin enzymes possess a catalytic C-terminal DxDxT motif and a C-terminal domain sequence (Fig.1.6) that is essential for PAP activity [160]. The DxDxT motif is required for PAP activity [160]. Lipin-1 and lipin-2 also contain a conserved serine residue separate from the active site that is required for catalytic activity [160].

The Lpin1^{1Hubr} rat contains a mutation in the proposed binding site of Mg^{2+} in the HADD IV domain due to a truncation in which exon 20 is missing and a frameshift that resulted in the inclusion of a 16bp segment of an intron [216]. The lipin-1 protein from Lpin1^{1Hubr} rats displays a complete loss of PAP activity and was associated with lipodystrophy and neuropathy [216]. The Lpin1^{1Hubr} mutation led to upregulation of CDP-diacylglycerol synthase-1 (Cds1) mRNA expression from post natal day 10 to 16 [216], which was not seen in *fld* mice [6]. Consistently, elevated levels of phosphatidylinositol (PI) were reported in Lpin1^{1Hubr} rats but PA accumulation was not reported. LPP activity in the Lpin1^{1Hubr} rat was upregulated compared to wild type rats and levels of ceramide and sphingomyelin were upregulated in the endoneurium of rats lacking Mg^{2+}-dependent PAP activity [216].

The fld^{2J} mouse contains a mutation of Gly48 to Arg in lipin-1. This point mutation is located in the regulatory NLIP domain of lipin-1 and causes decreased PAP activity and symptoms of lipodystrophy. Lipin-1 was unable to translocate to the nucleus in cells from fld^{2J} mice. Mutations in either of the catalytic aspartate residues resulted in complete loss of PAP activity [160]. Lipin-1 deficiency in mice causes lipodystrophy [154]. However, human subjects with mutations in lipin-1 do not show lipodystrophy and children who lack lipin-1 expression do not show lipodystrophy or deficits in formation of mature adipose tissue [217]. Thus the biology of lipins highlights the growing evidence for redundancy of function between the lipins. Overexpression of lipin-1 in adipose tissue of transgenic mice is associated with decreased adiposity and increased

sensitivity to insulin which is not associated with upregulation of adipogenic gene expression [218]. In contrast, overexpression of lipin-1 in skeletal muscle is associated with obesity and development of insulin resistance. Lipin-1 is also highly expressed in skeletal muscle and adipose tissue in mice.

Moreover, each lipin isoform shows a differential expression in mouse and human tissues. Intriguingly, peripheral neuropathy is not seen in young children with mutations in lipin-1 and this may be due to higher specific PAP activity of lipin-2 and lipin-3 in human peripheral nerves. In contrast, a small group of children with recessive mutations in lipin-1 were found to be susceptible to acute myoglobinuria and showed recurrent episodes of muscle weakness [219]. Lipin-1 is required for PAP activity in skeletal muscle and the only documented occurrence of PA accumulation in humans with mutations in lipin-1 is that of one individual with a null mutation in lipin-1 who was found to have PA accumulation in skeletal muscle, indicating this is a rare occurrence [219]. These mutations demonstrate that PAP activity is required for signalling pathways [219]. This data from human studies strongly emphasizes the dependence of PA accumulation on the specific PAP activity of lipin-1 compared to the PAP activity of lipin-2 and lipin-3, which varies according to cell and tissue type.

1.4.12. Association of lipin with the epidermal growth factor receptor

Dysregulated EGFR signalling is implicated in signalling pathways that mediate chemo-resistance in breast cancer. Increased EGFR expression is associated with resistance to chemotherapeutic treatment and relapse in breast cancer patients [220]. The EGFR has been specifically targeted by small molecule tyrosine kinase inhibitors including gefitinib and erlotinib and the monoclonal antibody cetuximab. However these drugs have shown less than expected benefit in breast cancer cell studies and in phase II clinical trials [221,222]. Studies with effectors downstream of EGFR activation such as breast cancer related kinase (Brk), suggest that increased understanding of these interactions is necessary to understand how to prevent EGFR activation, including ligand-independent activation of the EGFR [220].

It has been reported that PAP activity due to lipins (PAP-1) activity is detected in immune complexes isolated with an anti-EGFR antibody. This association of Mg^{2+}-dependent PAP activity with the EGFR is abrogated following treatment of cells with EGF [223]. Interaction with the EGFR represents an alternate mechanism by which lipins could be involved in regulation of signalling through the PLD pathway (Fig. 1.7). Mg^{2+}-dependent PAP activity was reported to dissociate from the EGFR and to simultaneously associate with PKCε at membranes in response to EGF treatment. Similarly, experiments in rat hepatocytes showed that treatment with EGF does not increase Mg^{2+}-dependent PAP activity but rather EGF stimulates PAP to dissociate from the EGFR and to interact with a different signalling complex at the membrane [122]. However, in

hepatocytes treatment with the epidermal growth factor (EGF), a growth factor which is known to activate PLD, did not stimulate Mg^{2+}-dependent PAP activity to translocate to membranes [122,224]. One of the lipin isoforms could translocate to membranes to associate with the EGFR but since membrane-associated PAP activity accounts for about 20% to 30% of total Mg^{2+}-dependent PAP activity [122], it remains unclear how soluble PAP could associate with the EGFR in the absence of growth factor stimulation as reported under basal levels of stimulation.

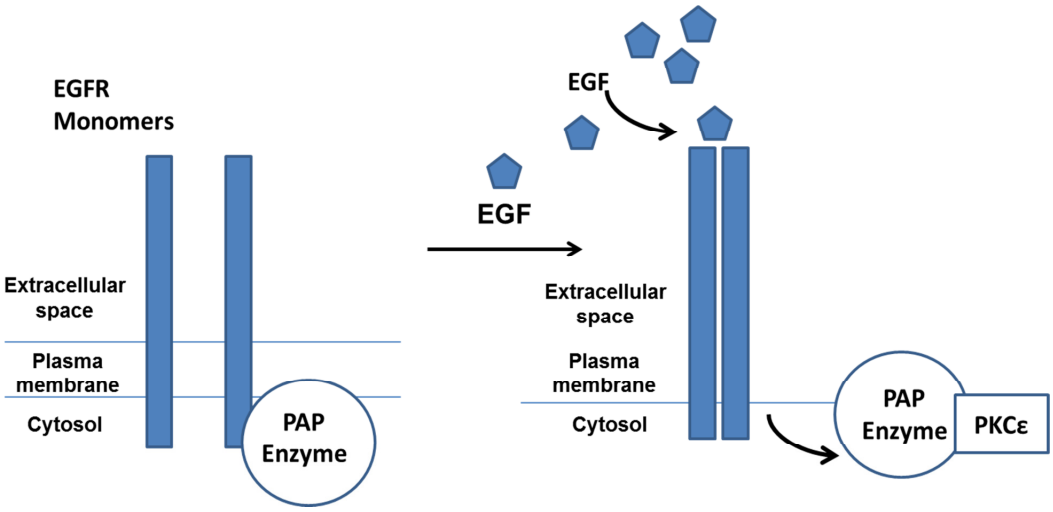


Figure 1.7. Interaction of PAP enzyme with the non-activated EGFR. Binding of EGF causes the PAP enzyme to dissociate from the activated and dimerized EGFR and to associated with PKCε in the membrane fraction of A431 cells as prepared by sedimentation coefficient centrifugation. The interaction of PAP with the EGFR is based on studies reported by Jiang *et al* [223]. The interaction of PAP enzyme specifically with the cytosolic domain of the EGFR is hypothetical and has not yet been demonstrated [223].

The PAP activity associated with the EGFR is reported to not be due to LPP activity, which was not found in the A431 cells in one study [223]. Although treatment of human dermal fibroblasts with EGF resulted in a rapid increase in the level of 1,2-DAG, the increased DAG was demonstrated to be due to activation of PLC rather than PLD activation [225]. However, a transient increase in PA caused by treatment of cells with propanolol, an inhibitor of PAP activity, was associated with increased endocytosis of the EGFR which might decrease the accessibility of the EGFR to bind to PAP-1 enzymes in the absence of ligand [95]. Endocytosis of the non-activated EGFR was also dependent upon PLD-derived PA [89]. It remains difficult to predict how the EGFR could be regulated by PAP since the EGFR is subject to many different levels of regulation. In cancer cells and other cell types, the EGFR exists in both low-affinity forms and high-affinity forms on the surface of cells. It is unknown whether this interaction is dependent on PLD activity and whether or not PAP acts directly with the EGFR or requires other binding partners. However, it is likely that an interaction of the lipins with the EGFR would be indirect since this interaction has not been identified in any studies of EGFR binding partners.

The EGFR interactome consists of many proteins and varies depending on cell type. Proteins that were found to bind to the phosphotyrosine residues of the EGFR in A431 cells and in breast cancer cells include Shc, Grb2, and STAT5, protein tyrosine phosphatase-2c (PTP-2c) (multiple binding sites) and Src, Cbl, Crk and SH3BGRL, which all possess SH2 or PTB domains [226,227].

None of these identified proteins are known binding partners of the lipins. Since all of the lipin isoforms can translocate to the nucleus, it is plausible that an interaction between EGFR and lipin and other binding partners might take place in the nucleus. EGFR can translocate to the nucleus where it may act as a transcription factor. The EGFR also binds to helicase A in cancer cells and this takes place in the nucleus [228,229]. EGFR accumulation in the nucleus may also contribute to cetuximab resistance in multiple cancer types. Nuclear transport of the EGFR may be dependent upon PKCε, which phosphorylates EGFR at T654, a residue which is part of the NLS of EGFR [230]. Nuclear EGFR has been shown to bind to STAT3, STAT5A, E2F1, PCNA, and DNA-dependent protein kinase (DNA-PK). Both STAT3 and STAT5A, which are confirmed to bind to the EGFR both by microarrays and by mass spectrometric analysis are transcription factors which bind to DNA sequences that are similar to those of genes regulated by lipin-1 [153]. Thus it would be of importance to understand the putative regulation of the EGFR by PAP enzymes.

1.4.13. Majeed syndrome and a role for lipin-2 in inflammatory signalling

The lipin isoforms likely perform distinct roles in physiology and metabolism. While lipin-1 deficiency is associated with decreased lipid droplet formation and increased activation of nuclear factor of activated T cells c4 (NFATc4), lipin-2 deficiency is associated with upregulated inflammatory signalling [231]. Mutations in lipin-2 occur rarely in humans. Majeed syndrome is

a very rare recessive disorder occurring in humans with a single amino acid substitution mutation in lipin-2 [160]. Majeed syndrome has provided insight into the role of lipin-2 in human biology [160]. Individuals with the Majeed mutation show a phenotype that includes inflammation in the skin and bones and anemia [232]. Paradoxically, lipin-2 expression is not found in skin or bone tissue, but is high in other tissues including brain, intestine, liver and kidney [160]. The Majeed mutation causes a loss of PAP activity but the transcriptional coactivator function is retained in the mutant protein [160]. Consistently, lipin-2 inhibits the effect of saturated fatty acids in promoting expression of inflammatory genes. Depletion of lipin-2 expression in macrophages resulted in activation of the c-Jun N-terminal kinase (JNK1) pathway [233]. Although PAP activity is little affected by knockdown of lipin-2 in some cells [160], lipin-2 expression was required for incorporation of fatty acids into TAG. Although PA accumulation is not reported in Majeed patients, knockdown of lipin-2 in macrophages stimulated the phosphorylation of ERK1/2 and JNK1 in response to stimulation with palmitic acid [233]. Treatment with the inhibitor PD98059 which blocks the ability of MEK1/2 to phosphorylate ERK1/2 or with a JNK1 inhibitor caused a decrease in the palmitate-stimulated expression of the pro-inflammatory cytokines IL-6 and ccl2 [233].

There is also an emerging role for lipin-1 in regulating inflammatory signalling. Evidence that low grade inflammation associated with obesity could be linked to lipin deficiency and increased fatty acid accumulation has emerged [234]. Recent studies in macrophages revealed that depletion of lipin-1 with

siRNA affected lipid droplet formation but did not decrease TAG formation [235]. The cytokine TNFα inhibits the expression of lipin-1 in a janus kinase-2 (JAK2)-dependent manner in 3T3-L1 fibroblasts [236].

1.4.14. Lipins regulate immune signalling

Stimulation of pro-inflammatory signalling in RAW264.7 and U937 macrophages with lipopolysaccharide (LPS) is associated with decreased cytosolic lipin activity and increased DAG levels on membranes of the endoplasmic reticulum [237,238]. Translocation of Mg^{2+}-dependent PAP activity was found in U937 macrophages, where LPS treatment caused a decrease in cytosolic PAP activity after 2 min of stimulation. This suggests that PAP translocates to membranes and hydrolyses PA, likely produced by PLD, to DAG. The increase in DAG was not only sensitive to *n*-butanol but it was also inhibited by D609, a PLC inhibitor. In addition to activation of DAG production by PLD signalling, DAG can transiently accumulate on the plasma membrane in response to activation of receptor tyrosine kinases coupled to phospholipase Cγ (PLCγ) or GPCR stimulation coupled to PLCβ activation [239]. In U937 leukemia cells, propranolol inhibited the formation of PA-derived DAG as stimulated by treatment with clinically relevant concentrations of the taxane drug docetaxel [240]. Propanolol is a fairly non-specific chemical inhibitor of PAP activity [123].

1.4.15. Lipins activate Group IVA phospholipase A_2 in a protein kinase C-dependent manner

Studies with pharmacological inhibitors suggest that Mg^{2+}-dependent PAP activity might be required for PKC-dependent activation of Group IVA PLA_2 (GIVA PLA_2) [241]. GIVA PLA_2 is a cytosolic PLA_2 that preferentially catalyzes the hydrolysis of membrane phospholipids with arachidonic acid at the *sn*-2 position [242]. Arachidonic acid can be converted to eicosanoids by lipoxygenase or cyclooxygenase (COX) enzymes in macrophages. The arachidonic acid release was sensitive to inhibition with ethanol, which suggested PLD might be required for eicosanoid production. Bromo-enol lactone (BEL) is widely regarded to be an inhibitor of both PAP and GIVA PLA_2 [243]. Treatment of amnionic WISH cells with BEL prevented the upregulation of COX2 expression in response to treatment with PMA. PLD was also required for the upregulation of COX2 expression since treatment with ethanol abrogated the effect of PMA stimulation of arachidonic acid release [237]. Activation of Toll-like receptor 4 (TLR4) with LPS caused an upregulation of COX2 that was also sensitive to inhibition by BEL or ethanol. These studies suggest that lipins are involved in immune signalling downstream of PLD activation. These pharmacological studies are supported by experiments in macrophages showing depletion of lipin-1 led to defective formation of lipid droplets and depletion of lipin-2 resulted in increased activation of JNK1 signalling [233,235].

1.4.16. Summary of evidence for and against a role of lipins in the phospholipase D pathway

There is no direct evidence suggesting that lipins are not regulators of the PLD pathway. In contrast, the ability of lipins to translocate to membranes where they could access intracellular PA derived from PLD suggests that these enzymes could be candidate regulators of PLD signalling [123]. Lipin-1 also translocates to the mitochondria in response to mitoPLD activation and PA accumulates in response to depletion of lipin-1 in the peripheral nervous system and in hearts of *fld* mice. Work with fluorescent biosensors showed that PA accumulates at the plasma membrane in response to PLD activation. However, it remains unknown whether lipins translocate to the plasma membrane to a significant extent in response to PLD activation. It also remains unknown whether depletion of lipins causes increased PA accumulation when the PLD pathway is activated. More work is needed to answer these remaining questions and to demonstrate a role for lipins in regulating PLD signalling.

1.5. Thesis Objectives and Hypothesis

The goal of this work was to investigate whether the lipins can convert PA to DAG in the PLD pathway. It has not yet been demonstrated whether PA accumulation is enhanced in lipin-depleted cells in response to PLD1/2 activation. In particular, LPA can promote breast cancer cell survival and signalling through activation of PLD [109]. Previous work has shown that PLD

signalling is one of the major contributing factors to breast cancer cell growth and progression [104,109]. Thus, it is important to understand how to attenuate PLD signalling in breast cancer. Recently, PLD chemical inhibitors were developed as a means to attenuate PLD. In order to attenuate PLD signalling, it is necessary to elucidate its downstream effectors. My hypothesis is that the lipins can regulate the effects of LPA-activated PLD signalling by translocating to the plasma membrane where the PAP activity can regulate the effects of LPA-activated PLD signalling by converting PA to DAG. We hypothesized that knockdown of lipin-1 would enhance PA accumulation when PLD was activated whereas overexpression of lipin-1 would attenuate PA-dependent signaling. We also hypothesized that lipin-1 would translocate to membranes when PA accumulates in response to PLD activation. Since PLD activity is elevated upon stimulation with PMA was observed in Doxorubicin-resistant MCF-7 cells [106] and doxorubicin resistance is positively correlated with increased PLD activity, we also hypothesized lipin-1 could control Doxorubicin resistance by regulating DAG levels potentially through NF-κB signaling. We furthermore hypothesized that the lipin-1 isoform could associate with the EGFR. We hypothesized that the lipin-1 isoform should co-immunoprecipitate with the EGFR in the absence of EGF stimulation. Furthermore, we proposed that lipin-1 should colocalize with the EGFR in a basal state of signaling but not after cell stimulation with a PLD agonist such as endothelin-1. We used chemical inhibitors along with genetic approaches to attempt to demonstrate a role for the lipins in regulating PLD signalling. The second part of this work involves a search for effectors of PLD signalling by investigating the hypothesis that lipins may regulate PLD signalling

via an interaction with the EGFR. Lipins may interact with the EGFR at the plasma membrane or may regulate EGFR function in the nucleus. For this section, we performed pulldowns of EGFR or flag-tagged lipins in cancer cell lines expressing high levels of EGFR in which flag-tagged lipin-1 or lipin-2 was overexpressed. We performed western blotting to test for co-immunoprecipitation of lipin-1 or lipin-2 with the EGFR and also determined PAP and LPP activity in EGFR immunoprecipitates. These experiments were followed up by preliminary studies to investigate whether lipin-1 can colocalize with EGFR using confocal microscopy.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

Plasmids for flag-tagged lipin-1β and flag-tagged lipin-2 and adenovirus vector expressing lipin-1β adenovirus and lipin-2 adenovirus were gifts from Dr. T. Harris (University of Virginia School of Medicine, Charlottesville, VA). 1-oleoyl-2-lyso-phosphatidic acid, human endothelin-1 and human epidermal growth factor (EGFR), 1,2-dioleoylphosphatidic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was fatty-acid free and purchased from MP Biomedicals (Solon, OH).

2.2. Cell Culture and Overexpression or Knockdown of Lipin-1, Lipin-2, and Phospholipase D Expression

2.2.1 Culture of cell lines

Doxorubicin-resistant and syngenic control MCF-7 cells were a gift from Dr. A. Parissenti (Laurentian University, Sudbury, ON). Doxorubicin-resistant cells were selected by being exposed to gradually increasing concentrations of doxorubicin until the selection dose reached 98.1 nM, which was the maximum dose tolerated by these cells as described previously [244]. The doxorubicin-resistant cells were maintained in 98.1 nM of doxorubicin in Dulbecco's Modified Eagle's Medium (DMEM) media supplemented with antibiotics penicillin, streptomycin, and amphotericin B. MDA MB 231 and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). MDA MB 231 cells were maintained in RPMI 1640 medium (Gibco) supplemented with

10% (v/v) fetal bovine serum (FBS) (GIBCO) and an antibiotic/antimycotic cocktail containing penicillin, streptomycin, and amphotericin B (Invitrogen Life Technologies, Carlsbad, CA). MCF-7 cells were maintained in DMEM medium (Gibco) supplemented with 10% (v/v) FBS and penicillin and streptomycin. All cell lines were grown in 5% CO_2 at 95% humidity and 37°C. A431 epidermoid carcinoma cells were a gift from Dr. M. Weinfeld, University of Alberta, Edmonton, Alberta.

2.2.2. Preparation of mouse embryonic fibroblasts from *fatty liver dystrophy* (*fld)* mice and control mice heterozygous for lipin-1

All animal protocols were approved by the University of Alberta Animal Policy and Welfare Committee. Mouse embryonic fibroblasts (MEFs) were prepared from *fld*/*fld* and *Lpin1*/*fld* mouse embryos. *Lpin1*/*fld* and *fld*/*fld* mice were bred and the females were monitored for vaginal plugs. At day 15 post-coitus, the pregnant female mice were sacrificed and the embryos were dissected from the uterus. For each embryo, the head was removed and discarded, the organs were removed with forceps and the embryo carcass was washed with HEPES Buffered Saline (HBS) three times to remove all remaining blood. Embryo carcasses were treated individually and were homogenized by chopping with a razor blade. The minced carcasses were digested with 0.05% trypsin-EDTA and 2% crude DNAse for 30 min at 37°C with shaking on an orbital shaker. The cells were then centrifuged to remove the trypsin and each digested embryo was placed in a 150 cm^3 flask that was coated with 0.1% gelatin (Gibco)

prior to the experiment. The MEFs were grown in DMEM supplemented with 10% FBS, 2 mM glutamate, and antibiotic cocktail at 5% CO_2 at 95% humidity and 37^{o}C. MEFs were passaged by trypsinization with 0.05% trypsin-EDTA. From P1 onwards, MEFs were grown in flasks without coating with gelatin. The MEFs were collected at P1 and genotyped for lipin-1, lipin-2, and lipin-3 expression by quantitative real time PCR (RT-PCR). MEFs were used for experiments up to and including P6.

2.2.3. Adenoviral overexpression of lipin-1 or lipin-2

Rat2 fibroblasts or MDA MB 231 cells were seeded and allowed to attach overnight. The cells were then infected with adenoviral vector for lipin-1β, lipin-2, or control vector for 36 hours. Cells were starved for 18 hours followed by harvesting in 250 mM sucrose buffered to pH 7.4 with $NaHCO_3$. Overexpression was demonstrated by RT-PCR or western blot.

2.2.4. Plasmid propagation and isolation

Plasmids for lipin-1β and for lipin-2 were propagated by transformation in Subcloning Efficiency DH5α competent *E.coli* according to the manufacturer's protocol (Invitrogen). Colonies from the transformation were selected and grown in LB Broth containing the relevant antibiotic for selection. Plasmid DNA was

isolated using a Miniprep kit (Qiagen). Plasmid DNA was quantified by spectrophotometry on a Nano Drop 2000 (Thermo Scientific).

2.2.5. Transient transfection of MDA MB 231 cells and A431 cells with lipin-1 or lipin-2 plasmid

Cells were seeded on 10 cm dishes in antibiotic-free media and allowed to attach for 24 h. Twenty µg of plasmid DNA were mixed with 700 µl of Opti-MEM (Gibco) and incubated for 5 minutes at room temperature. Then, 30 µl of Lipofectamine 2000 (Invitrogen) were mixed with 700 µl of Opti-MEM and incubated for 5 minutes at room temperature. Plasmids were mixed with Lipofectamine 2000 (Invitrogen) and incubated for 20 minutes at room temperature. Mixtures were then added dropwise to the dishes. After 18 hours, the media was removed and replaced by regular media. After 24 hours, the cells were collected for immunoprecipitation.

2.2.6. Knockdown of lipins with siRNA

Cells were seeded on 3.5 cm or 6 cm dishes in antibiotic-free media and allowed to attach for 24 hours. At 50% confluence, cells were transfected with double-stranded *Smartpool* small interfering RNAs (siRNAs) for human lipin-1, lipin-2, lipin-3, Cy3-conjugated non-targeting control (siGLO), or non-targeted controls (Dharmacon, Inc. Lafayette, CO). The transfection mixture was added in a dropwise fashion to each dish containing 1 ml of Opti-MEM. The final

concentration of Lipofectamine 2000 in each transfected dish was 1.41 µg/ml. When the tansfection reagent used was INTERFERin™ (Polyplus Transfection, Cedex, France), the final concentration in each transfected dish was 0.5 ug/ml of INTERFERin™. The final concentration of siRNA in each dish was 50 nM of total siRNA and 12.5 nM of each individual siRNA of the set of 4 that were combined. Plasmids or siRNA were mixed with Lipofectamine 2000 (Invitrogen) and incubated for 20 minutes at room temperature. Cells were incubated with siRNA for 24 hours and the media was changed after 24 hours to regular media. INTERFERin™ transfection reagent was optimized for knockdown of lipins from a concentration response curve (Fig. 2.1).

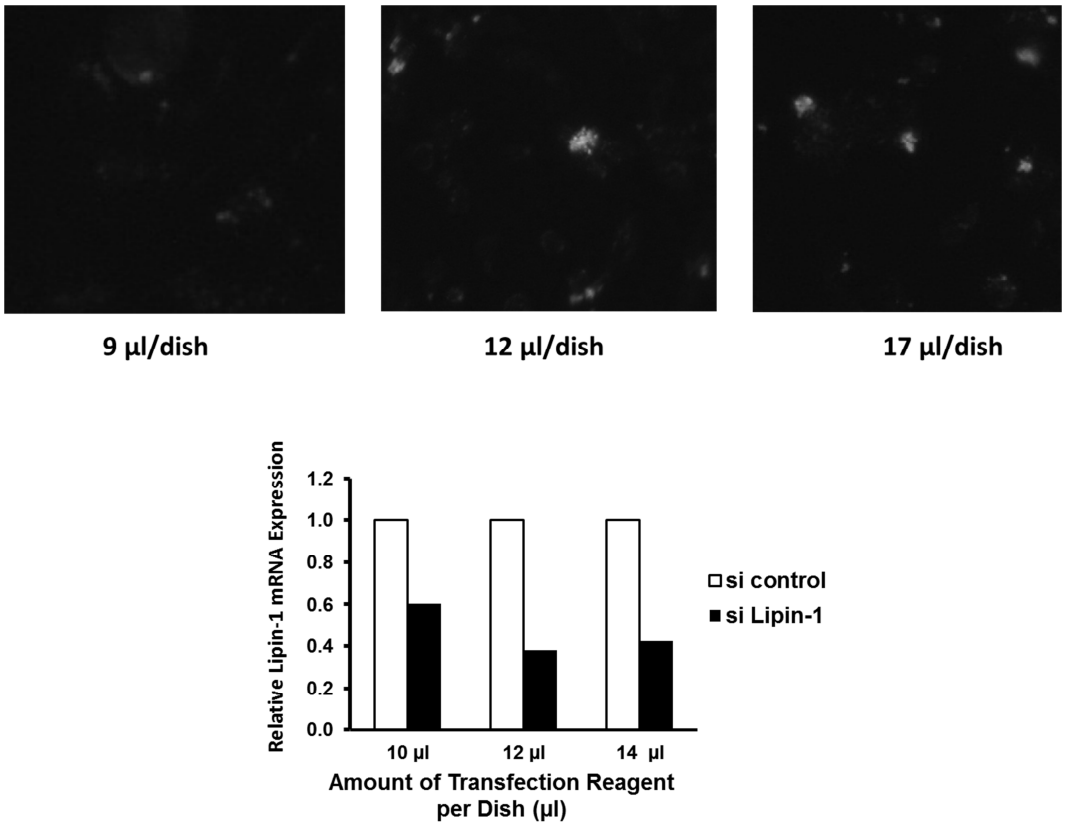


Figure 2.1. Optimization of siRNA-mediated knockdown of lipin-1 with INTERFERin™ transfection reagent in MDA MB 231 cells by siRNA. INTERFERin™ transfection reagent was also optimized using transfection with siGLO (Dharmacon) at 25 nM per 35 mm dish of cells. This figure illustrates a representative experiment. Lipin-1 expression was normalized to cyclophilin-A mRNA expression.

2.2.7. Adenoviral overexpression of phospholipase D

Rat2 fibroblasts or MDA MB 231 cells were seeded and allowed to attach overnight. The cells were then transfected with adenovirus for lipin-1β, lipin-2, or vector for 36 hours. Cells were starved for 18 hours followed by harvesting in sucrose buffer containing 250 mM sucrose and overexpression was demonstrated by western blotting.

2.3. Quantification of lipin-1, lipin-2, lipin-3, and phospholipase D expression and activity

2.3.1. Quantitation of mRNA expression of lipins by quantitative Real Time-PCR

Cells cultured on dishes were rinsed twice with ice-cold HBS and scraped into lysis/binding solution from an RNaqueous® kit (Ambion Inc. Austin, TX). Lysates were homogenized by passing several times through a sterile 26-gage needle. mRNA was isolated using an RNAqueous kit and DNA was removed from the RNA using a DNA-free kit (Ambion) according to the manufacturer's instructions. mRNA was reverse transcribed to cDNA using Superscript II reverse transcriptase, dNTPs, random primers, and RNAout and these reagents were purchased from Invitrogen. Quantitative real time PCR (qRT-PCR) was performed using 0.2 µM of each primer per reaction to amplify genes in each RT-PCR reaction. Primers sequences are reported in Table 1. Standard curves were prepared for each pair of primers. Samples and standard curves were analyzed in triplicate on a 7500 quantitative Real Time PCR Cycler (Applied Biosystems, Foster City, CA) using Sybr Green as the fluorescent detection dye (Quanta). Gene expression was quantified according to the Pfaffl method [245]. The gene expression was normalized according to the expression of the housekeeping gene cyclophilin A.

Table 1. Primer sequences used in RT-PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Ref
Lpin1α Mouse	GGTCCCCCAGCCCCAGTCCTT	GCAGCCTGTGGCAATTCA	[246]
Lpin1β Mouse	CAGCCTGGTAGATTGCCAGA	GCAGCCTGTGGCAATTCA	[246]
Lpin2 Mouse	TAGATGCAGACCCTGTTCCC	CTGGTGCTGGCTTCTTTGT	[199]
Lpin3 Mouse	AAAGACTGGACACACCAGGG	TGCTGGATATCACTCAGGCA	[199]
Lpin1 Human	GTTTGGGGCTGTGTGTGAAACCTA	CCCCACTGTGTTCAGAAG	
Lpin2 Human	CCCCACTGTGTTCAGAAG	TTGTCTCTGCATCCGACAAG	
Lpin3 Human	CAAGGCCACCATCTACCTGT	CCCTGGTGTGTCCAGTCTTT	
CypA Human	TTCATCTGCACTGCCAAGAC	TCGAGTTGTCCACAGT	[247]
CypA Mouse	CACCGTGTTCTTCGACATCAC	CCAGTGCTCAGAGCTCGAAAG	[142]
COX-2 Mouse	TGTATCCCCCCACAGTCAAAGA	ACCAGACCAAAGACTTCCTGCC	
NF-κB p50 Mouse	AAAATGCCCCACGGTTATGG	GGACGATGCAATGGACTGTCA	

Primer sequences that are not referenced were designed using the default parameters in Primer Express 2.0 software. Abbreviations are as follows: Lpin1, lipin-1; Lpin2, lipin-2, Lpin3, lipin-3; CypA, cyclophilin A.

2.3.2. Western blotting for lipin and phospholipase D expression

Cell lysates were collected in modified RIPA buffer containing 50 mM HEPES pH 7.4, 0.1% SDS, 150 mM NaCl, 1% Igepal CA-630, 2 mM $MgCl_2$, and 0.5% sodium deoxycholate and EDTA-free protease inhibitor cocktail (Sigma) and microcystin-LR (Cedarlane Labs, Burlington, ON). Microcystin-LR was used to inhibit the activity of protein phosphatase-1, which can phosphorylate and inhibit the PAP activity of the lipins. Protein concentrations of cell lysates were determined by Bicinchinonic (BCA) Assay (BioRad) as per the manufacturer's directions using a standard curve prepared with BSA. Protein lysates were added to a 96-well plate and the absorbance at 700 nM was measured and a concentration curve was constructed and used to determine the protein concentrations. Lysates were added to sample loading buffer (Tris-Cl/SDS, 10% β-mercaptoethanol, glycerol) and heated for 10 min at 95^{o}C. Samples were loaded onto SDS-PAGE gels and electrophoresis was performed in Tris-glycine buffer. The proteins in the gels were transferred onto nitrocellulose at 4^{o}C overnight at 400 mA using a Transblot transfer apparatus (BioRad). Blots were dried for 20 minutes followed by blocking for two hours at room temperature in Odyssey^{TM} Blocking buffer: Phosphate Buffered Saline (PBS) 1:1. Blots were incubated overnight in primary antibodies, washed 3 times for 10 minutes each and incubated in IR Dye 680 goat anti-mouse and IR Dye 800 goat anti-rabbit secondary antibodies (Mandel Scientific) at a dilution of 1:5000 for each secondary antibody at room temperature for 45 minutes. Dilutions for primary antibodies are reported in Table 2. Blots were rinsed and scanned on an

Odyssey™ Infrared Imaging System (LI-COR Biosciences). Samples which were blotted for PLD1 antibodies were not heated prior to separation by SDS PAGE. The epidermal growth factor receptor (EGFR) was detected with an antibody against the C-terminal region (Santa Cruz Biotechnology, CA) and lipin-1β was detected by an antibody against the C-terminus (a gift from Dr. T. Harris, University of Virginia). Recombinant HA-tagged lipin-1 was detected with anti-HA antibody (Covance Laboratories,CA). Blots were analyzed by densitometry using ImageJ and intensity values were normalized to the signal obtained from blotting for anti-calnexin antibody (Stressgen) or anti-α-tubulin antibody (Sigma). Lipin-2 was detected by enhanced chemiluminescence. Lipin-2 blots were blocked in milk for 1 hour at room temperature followed by incubation in primary antibody in 1% milk at 4°C overnight. Blots were incubated in secondary antibody for 90 min at room temperature followed by detection with horse radish peroxidase (BioRad, Hercules, CA).

Table 2. Primary antibodies used for immunoblotting or co-immunoprecipitation

Antibody Host Species	Target	Western Blotting Dilution	Co-Immunoprecipitation Dilution	Supplier
Rabbit polyclonal	EGFR	1:1000	1:50	Santa Cruz Biotechnology
	Lipin-1 C-terminus	1:500		Dr. T. Harris, University of Virginia
	Lipin-2	1:500		Genscript
	Calnexin	1:2000		Stressgen
	RhoA		1:50	Upstate
Mouse monoclonal	Flag	1:1000	1:75	Clontech Laboratories, CA
	HA	1:1000	1:75	Covance
	α-tubulin	1:1000		Sigma Aldrich
	GAPDH	1:2000		Sigma Aldrich

Table 3. Antibodies used for immunofluorescence

Antibody Host Species	Target	Dilution	Supplier
Primary Antibodies			
Rabbit polyclonal	EGFR	1:50	Santa Cruz Biotechnology
Mouse monoclonal	HA	1:200	Covance
Secondary Antibodies			
Donkey anti-rabbit	Alexa Fluor 647	1:300	Molecular Probes, OR
Donkey anti-mouse	Alexa Fluor 555	1:300	Molecular Probes

2.3.3. Measurement of phospholipase D activity

Rat2 fibroblasts or MEFs were seeded and allowed to attach for 24 hours. Cells were starved for 10 hours in serum-free DMEM with 0.1% BSA and Pen/Strep for 10 hours. Cells were then labelled with 4 µCi per dish of [9,10-^{3}H]-palmitate (Perkin-Elmer Life Sciences, Boston, MA) for 2 hours at 37^{o}C. Cells were then washed with serum-free DMEM containing 0.1% BSA and Pen/Strep. Cells were pre-incubated in 1-butanol or vehicle for 30 minutes followed by addition of 10uM LPA, endothelin-1 or control treatment for 5 minutes. Cells were then collected by scraping into methanol and lipid extraction was performed by sequential addition of chloroform and 2M KCl/10 mM HCl, followed by centrifugation. The upper protein phase was removed and solvent was

evaporated under nitrogen and redissolved in chloroform: methanol 9:1 and loaded onto Silica Gel-60 plates (Merck, Darmstadt, Germany) and separated by thin layer chromatography (TLC). TLC plates were developed in the upper phase of the ethylaceate/iso-octane/acetic acid/water (130:20:30:100 by vol.) solvent mixture. Plates were stained with I_2 and the PC, PE and phosphatidylbutanol (PB) bands were identified by comparison with standards and scraped into scintillation vials and measured by liquid scintillation counting on a beta counter. Relative PLD activity was determined as the percentage of [^{3}H]-PB relative to the labelling in PC and PE.

2.3.4. Phosphatidic acid phosphatase (PAP) activity assay

Cells were lysed in 250 mM sucrose, adjusted to pH 7.5 with $NaHCO_3$, with 2 mM DTT, protease inhibitors, and 100 nM Microcystin-LR and sonicated. Protein concentrations of cell homogenates were determined by BCA Assay. Cell lysates were incubated in 100 mM Tris-maleate buffer with 5 mM $MgCl_2$ at pH 6.5 or pH 7.4, in the presence of 1 mM tetrahydrolipstatin (from Dr. M. Meier, Hoffman La-Troche, Switzerland) to inhibit the activity of lipases to hydrolyze the DAG product and 20 µL of PAP substrate. The PAP substrate contained [^{3}H]-PA prepared from [^{3}H]-palmitate and mixed in a molar ratio of 3:2 with non-radioactive PC and 1mg/ml FA-free BSA prepared according to Martin *et al* [248]. Samples were quantified using the PAP assay and each assay contained the final concentration of 100 mM Tris-maleate, pH 6.5 or pH 7.5, 2.5 mM $MgCl_2$, 1 mg/ml BSA, 0.6 mM [^{3}H]-PA and 0.4 mM non-radioactive PC. Each assay

contained approximately 1 x 10^5 dpm. The amount of cell lysate included was used to ensure that the conversion of PA to DAG accounted for less than 10% of the PA added. The rate of PAP reaction was measured at three different concentrations of protein in order to demonstrate the proportionality of the activity to the amount of protein and to allow calculation of the relative rate of PAP activity per sample. The PAP assay was optimized to measure the formation of DAG from labelled PA in order to maximize the contribution of PAP activity relative to LPP activity. Lysates were incubated in parallel in the presence and absence of 8 mM N-ethylmaleimide (NEM), an inhibitor of PAP activity, in order to determine the contribution of LPP enzymes to the total PA-phosphohydrolase activity. The activity due to LPPs was subtracted from the total activity in order to obtain the PAP activity in the sample. Samples were incubated for with shaking for 2 hours at 37°C. The reaction was then quenched by extraction of [^{3}H-DAG] with 2.2 ml of chloroform:methanol (95:5, by vol.) and basic alumina was added to adsorb PA. The alumina was collected by centrifugation and 1 ml from each sample of the organic phase was transferred to scintillation vials and evaporated by heating in a boiling water bath for 10 minutes. A hair dryer was used to evaporate remaining solvent. [^{3}H]-DAG was determined by liquid scintillation counting.

2.3.5. Measurement of lipid phosphate phosphatase (LPP) activity by the triton-micelle assay

The LPP activity assay used was the mixed triton micelle assay. Cells were starved for 10 hours and lysed and scraped into buffer containing 250 mM sucrose with 2 mM DTT and protease inhibitors. Equal amounts of cell lysate were added to LPP buffer containing 100 mM Tris-Maleate, pH 7.4, 8 mM NEM and 1 mM EDTA and 1 mM EGTA. Triton-X-100 was used to solubilize PA and it also extracts lipids and integral membrane proteins from lysates. The LPP assay substrate contains PA in Triton-X-100 micelles and maximizes LPP activity relative to PAP activity. The amount of protein and incubation were done so that the amount of PA hydrolyzed per sample did not exceed 10%. The activity was measured at 3 different protein concentrations to validate the method by ensuring that the amount of homogenate in the assay was proportional to the activity measured. The reaction was started by adding substrate to a final concentration of 0.83 Ci/mol of [^{3}H]-PA and 0.6 mM Triton-X-100. Samples were incubated for 30 minutes with shaking at 37^{o}C. The reaction was then quenched by addition of 2.2 ml of chloroform:methanol (95:5, by vol.) and unreacted PA was adsorbed by adding basic alumina and vortexing. The alumina was collected by centrifugation and 1 ml from each sample of the organic phase was removed and dried down by immersion in a boiling water bath for 10 minutes. A hair dryer was used to evaporate remaining solvent. [^{3}H]-DAG was determined by liquid scintillation counting.

2.4. Translocation of Lipin-1 and Lipin-2 to Membranes

2.4.1. Treatment of cells with phospholipase D agonists and inhibitors

Cells were seeded and allowed to attach for 24 hours. Cells were starved for 10 hours in serum-free DMEM with 0.1% BSA and Pen/Strep. Cells were pre-incubated in PLD inhibitors, the calcium-dependent DAG kinase isoform inhibitor 3-[2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl]-2,3-dihydro-2-thioxo-4(1H)quinazolinone (R59949), or vehicle for 30 minutes followed by addition of agonists including LPA or endothelin-1 or vehicle with inibitor for 5 minutes or over a timecourse. Cells were then collected by digitonin lysis as described in the following section.

2.4.2. Digitonin lysis and fractionation of cells

Cells were washed 3 times with ice-cold HBS and ice-cold digitonin lysis buffer containing 10 mM HEPES, pH 7.4, 0.5 mM DTT, 0.081 mM digitonin, protease inhibitor cocktail, and 100 nM microcystin-LR was added. The concentration of digitonin used was based on a digitonin concentration curve (Fig. 2.2). The dishes were incubated on ice for 6 minutes to allow selective permeabilization of membranes. The lysis buffer and released fraction was collected and the dish was washed three times with ice-cold HBS. The cell ghosts were scraped from the dish into modified RIPA buffer prepared as described in Section 2.8. The cytosolic fraction was centrifuged at 10,000 rpm

for 5 minutes to pellet any intact cells that may have detached from the dish during digitonin lysis. The supernatant was collected and the pellet discarded. The membrane fraction was sonicated at 45% output on ice for 7 seconds, repeated once. The membrane fraction was then centrifuged at 10,000 rpm for 5 minutes to pellet insoluble material and the supernatant was collected.

2.4.3. Lactate dehydrogenase assay to verify separation of cellular fractions by digitonin lysis

Cytosolic and membrane fractions collected by digitonin lysis were loaded into a 96-well plate. A cocktail containing 1 M Tris pH 7.4, sodium pyruvate, and NADH was added to the samples and immediately kinetic Vmax absorbance readings at 340 nm were taken on an EAR 340 SLT plate reader (SLT Lab Instruments, Austria) for a total time of 30 minutes at 20 second intervals. The lactate dehydrogenase (LDH) activity in each fraction was calculated. Values of 85-95% of activity in the cytosol indicated a successful separation of cytosol and membrane fractions.

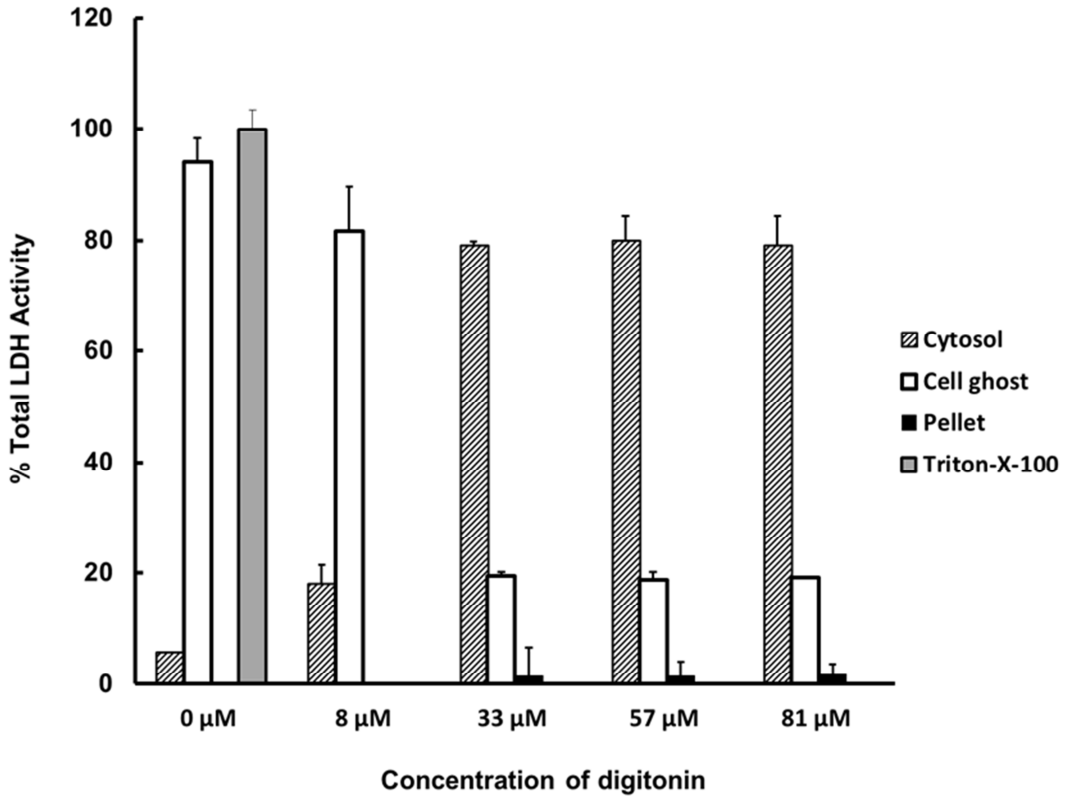


Figure 2.2. Optimization of digitonin lysis in MDA MB 231 cells. Values represent means ± ranges.

2.5. Cell Migration Assay

Rat2 fibroblasts were seeded in 10 cm dishes in DMEM with 10% FBS and antibiotics at a density of 1 million cells. After 24 hours, the media was replaced with starvation media consisting of DMEM with 0.4% bovine serum albumin and antibiotics without FBS. Starvation was allowed to proceed for 15 to 18 hours. Meanwhile, transwell inserts with 0.8 µM pore size (Corning, Inc,) were coated with 0.143 mg/ml bovine fibronectin (Sigma) and left overnight to dry

in a sterile hood. After 15 hours, the cells were washed twice with HBS and lifted from the plate with trypsin-EDTA. After cell detachment, the trypsin was inhibited by addition of 0.1% soybean trypsin inhibitor (Sigma) in HBS and additional starvation media was then added. The cells were centrifuged for 10 minutes at 1000 rpm and the supernatant was removed and the cells were resuspended in starvation media. The cells were counted and 150,000 cells were seeded per well. Cells were allowed to attach for 2 hours, then agonist was added to the bottom well. The transwells were placed in the outer wells and the plates were incubated for 6 hours at 37°C. Migration was stopped by removing the transwells from the outer wells, removing the media and remove the cells attached to the upper surface of the filters with a cotton swab. The cells were fixed by placing the transwells into 4% formaldehyde (Sigma) for 1 hour at room temperature. The transwells were then stained with 1 µg/ml Hoescht 33258 for 2 hours to stain nuclei. The transwells were rinsed twice in HBS and cells on the lower surface of the transwells were counted on a fluorescence microscope (Leica DM IRB). For quantification of migration, 10 random fields were counted per transwell.

2.6. Quantification of phosphatidic acid and diacylglycerol

2.6.1. Lipid labelling

Cells were seeded and allowed to attach for 24 hours. Cells were starved for 10 hours in serum-free DMEM with 0.1% BSA and Pen/Strep for 10 hours.

Cells were then labelled with [9,10-^{3}H]-palmitate for 2 hours at 37^{o}C. Cells were then washed with serum-free DMEM containing 0.5% BSA and Pen Strep. Cells were pre-incubated with inhibitors including VU0155056, a pan PLD inhibitor, VU0359595, a PLD1 isoform-specific inhibitor, VU0285665-1, a PLD2 isoform-specific inhibitor, vehicle for 30 minutes. All PLD inhibitors were purchased from Avanti Polar Lipids (Alabaster, AL). PLD agonists, including LPA or endothelin-1, or control treatment were then added. Cells were collected by scraping into methanol and lipid extraction was performed by sequential addition of chloroform and 2 M KCl/10 mM HCl, followed by centrifugation. The upper protein phase was removed and solvent was evaporated under nitrogen and redissolved in chloroform: methanol 9:1 and loaded onto Silica Gel-60 plates and separated by TLC. Internal standards of 30 nmoles of di-oleoyl phosphatidic acid (Sigma Aldrich) or 70 nmoles of PC (Sigma Aldrich) were loaded on each sample lane. To resolve PA, TLC plates were developed twice in chloroform/methanol/ammonium hydroxide (65:35:7.5 by vol.) solvent mixture. Plates were dried and cut between PC and PA. The plates with PA bands were then turned upside down and developed in chloroform/methanol/acetic acid/acetone/water (50:10:20:12:5, by vol.) to separate acidic phospholipids. To resolve diacylglycerol and neutral lipids, TLC plates were developed in hexane/diethyl ether/acetic acid. Plates were stained with iodine and the PC and PA bands were identified by internal standards and scraped into scintillation vials. Counts were measured by liquid scintillation counting on a scintillation counter.

2.6.2. Phosphatidate mass assay

MEFs were seeded on 10 cm dishes and allowed to attach for 24 hours. Cells were starved for 10 hours in serum-free DMEM with 0.1% BSA and Pen/Strep for 10 hours Cells were pre-incubated with PLD chemical inhibitors including VU0155056, VU0359595, or, VU0285665-1, a PLD2 isoform-specific inhibitor, vehicle for 30 minutes. PLD agonists including LPA, endothelin-1 or control treatment were then added. Cells were collected by scraping into methanol and lipid extraction was performed by sequential addition of chloroform and 2 M KCl/10 mM HCl, followed by centrifugation. The upper protein phase was removed and solvent was evaporated under nitrogen and redissolved in chloroform: methanol 9:1 and loaded onto Silica Gel-60 plates and separated by TLC. An internal standard of di-oleoyl phosphatidic acid was loaded on each sample lane. To resolve PA, TLC plates were developed in chloroform/methanol/ammonium hydroxide (65:35:7.5 by vol.) solvent mixture and developed twice. Plates were dried and then turned upside down and developed in chloroform/methanol/acetic acid/acetone/water (50:10:20:12:5, by vol.). The plates were dried and then stained for 1 hour with 0.03% Coomassie Brilliant Blue R250 (BioRad) in 20% methanol and 100 mM NaCl with shaking at room temperature. The plates were destained with 20% methanol for 15 minutes followed by scanning on the Odyssey^{TM} Infrared Imaging System at 700 nm. Destaining was repeated until a low background was achieved. The Odyssey^{TM} densitometry software was used to quantify the bands representing PA and a standard curve was prepared to quantify the mass of PA from the phosphate

assay as shown in Fig. 2.3. The amount of PA in experimental samples was determined by comparison with the standard curve for PA.

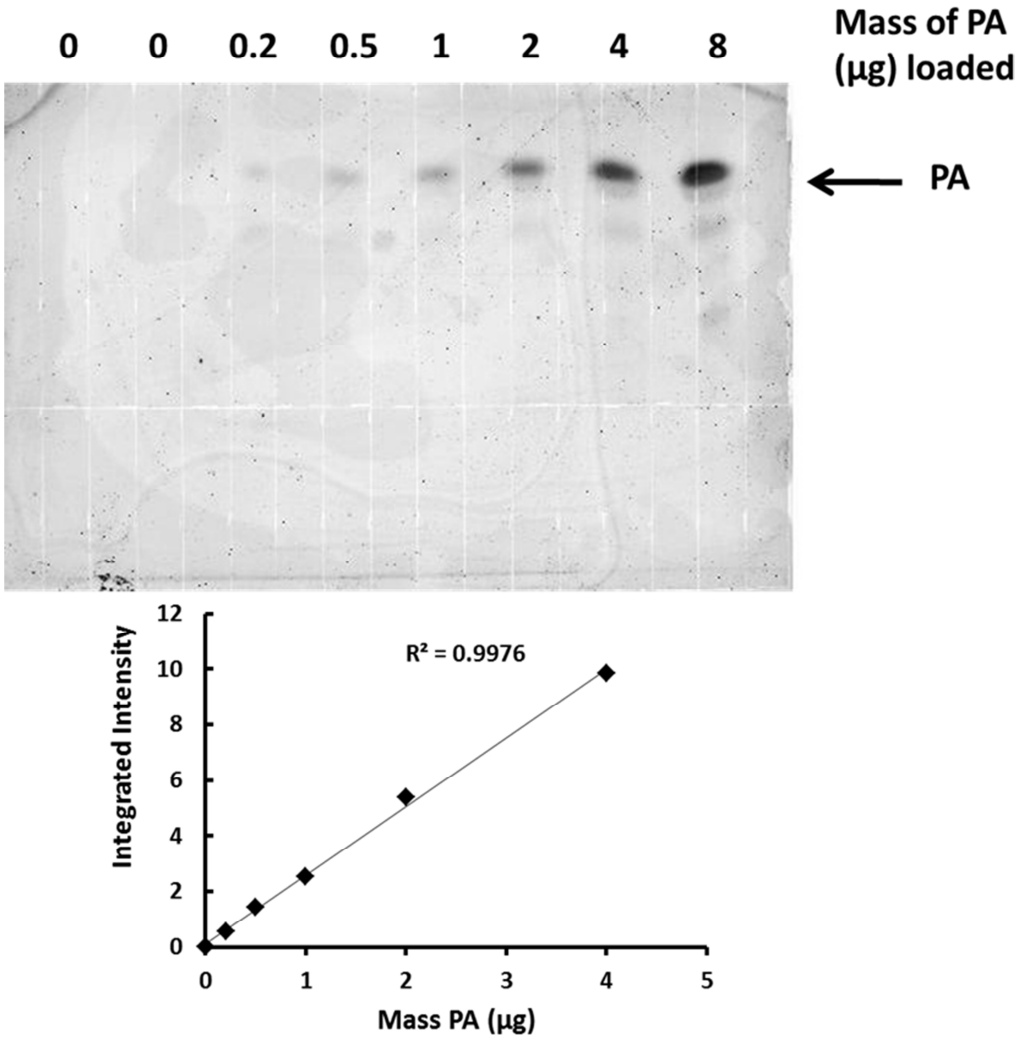


Figure 2.3. Measurement of the linear range for the assay used for detection of bulk cellular PA. A representative standard curve prepared from the PA band intensity is shown on the TLC plate. PA mass is visualized by Coomassie Brilliant Blue staining. The standard curve was calculated using the integrated density software in the OdysseyTM program.

2.6.3. Phosphate mass assay

Total phospholipid in samples was determined by preparing samples and a standard curve from 1 to 100 nmol of glycerol-3-phosphate in water. Samples were evaporated under nitrogen and 55 µl perchloric acid was added. The samples were heated to 180°C for 30 minutes in order to hydrolyze the organic phosphate to inorganic phosphate. Samples were cooled to room temperature and 278 µl of water was added. To each sample was then added 55 µl of 2.5% ammonium molybdate and 55 µl of freshly prepared 10% ascorbic acid. The samples were heated in a water bath at 90°C for 15 minutes. Half of the final volume was added to wells of a 96-well plate in duplicate and absorbance at 700 nm was measured with an Easy Reader EAR 340.

2.7. Immunoprecipitation of Lipin-1 and Lipin-2

2.7.1. Cell culture and chemical crosslinking

MDA MB 231 or A431 cells were seeded on 10 cm dishes. At 50% confluence, cells were transfected with plasmids as described in Section 2.2.5. After 24 hours of transfection, cells were starved for 6 hours with media containing 0.1% BSA followed by treatment with EGF or vehicle. Cells were then not treated or treated at room temperature with chemical crosslinkers. Cells were treated with 2 mM disuccinimydyl propionate (DSP) for 30 minutes according to the manufacturer's directions (Thermo Scientific), followed by 15

minutes of quenching with 20 mM Tris-HCl, pH 7.5. Cells were also crosslinked with 4% paraformaldehyde for 20 minutes, followed by quenching for 20 minutes with 1 M Tris-HCl, pH 7.5. Cells were then treated with vehicle or EGF for 5 minutes. Cells were permeabilized by digitonin lysis for 5 min on ice to separate membranes from the cytosol and the cytosol fraction was collected. The membrane fractions were scraped into buffer containing 1% Triton-X-100, 20 mM Tris-HCl, pH7.3, 150 mM NaCl, 1 µM ZnCl, 5 mM Na_2HPO_4, 1 mM EDTA, 1 mM EGTA, 400 µM sodium orthovanadate, protease inhibitor cocktail and 100 nM microcystin-LR. Samples were lysed by rocking for 1 hour at 4°C. Samples were centrifuged to pellet insoluble material and protein was determined.

2.7.2. Immunoprecipitation of lipin-1 and lipin-2

Membrane fractions were pre-cleared for 1 hour by incubation with protein G sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden). Samples were centrifuged and the supernatant was collected and transferred to a new tube. The pre-cleared lysate was incubated with anti-flag (Clontech), anti-EGFR (Santa Cruz), or anti-Rho A (Upstate) antibodies overnight at 4°C. Protein G sepharose beads were then added and the samples were incubated overnight at 4°C. Samples were then centrifuged and the supernatant was removed. The beads were washed 3 times for 3 minutes on the rotating shaker at 4°C with binding buffer. Sample loading buffer was then added to the beads and the beads were boiled for 5 minutes and collected by centrifugation. The protein

sample bound to the beads was loaded onto a 6% SDS-PAGE gel and resolved by gel electrophoresis and analyzed by western blotting.

2.7.3. Measurement of phosphatidic acid phosphatase (PAP) activity in epidermal growth factor receptor immunoprecipitates

Immunoprecipitation of tagged lipin-1 or tagged lipin-2 was performed on duplicate samples in buffer containing 1% Triton-X-100, 20 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1 µM ZnCl, 5 mM Na_2HPO_4, 1 mM EDTA, 1 mM EGTA, 400 µM sodium orthovanadate, protease inhibitor cocktail and microcystin-LR, with the addition of 0.5 mM DTT. After incubation of beads with protein lysates and antibody, the beads were washed once with immunoprecipitation buffer for 3 minutes on an orbital shaker at 4°C. Dilutions of antibodies are given in Table 2. The beads were then washed twice for 3 minutes with 1 mL of buffer containing 300 mM sucrose adjusted to pH 7.4 with $KHCO_3$, 0.5 mM DTT, protease inhibitors and 100 nM microcystin-LR. All of the supernatant was removed and the beads were incubated in PAP assay buffer at a final concentration of 1.5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 7.5. Another set of samples was incubated in this buffer with the addition of 8 mM NEM in order to measure NEM-insensitive activity. To the beads was added 20 µl of 300 mM sucrose adjusted to pH 7.4 with 250 mM $KHCO_3$, and 0.5 mM DTT, and 20 µl of PAP substrate. Reactions with and without NEM were analyzed by the PAP assay with the modification that samples were incubated for 6 hours at 37°C as this time was determined to give optimal PAP activity in the pulldown.

2.7.4. In gel tryptic digestion and protein identification by mass spectrometry

MDA MB 231 cells were transfected with flag-lipin-1 plasmid or not transfected as a control. Cells were either treated with DSP or not treated and lysed by digitonin lysis. Co-immunoprecipitation was performed on equal amounts of protein from the membrane fractions. Samples that were coimmunoprecipitated with anti-flag antibody were separated by gradient SDS-PAGE gel electrophoresis. Gels were stained by silver stain using Silver Stain Plus kit (BioRad) according to the manufacturer's protocol. Bands of interest were excised from the gel and submitted for analysis by LC MS/MS mass spectrometry at the IBD Facility, University of Alberta.

2.8. Indirect Immunofluorescence of Lipins and Confocal Microscopy

Circular coverslips (Fisher Scientific) were sterilized by autoclaving. MDA MB 231 cells were seeded on coverslips in 12-well plates (Corning, Inc.) and allowed to attached overnight. The cells were either not transfected or transfected with vector or adenovirus for HA-lipin-1 or transfected with plasmid as described in section. Cells were then treated with immunofluorescence at room temperature as described. After 24 hours of transfection, coverslips were washed with HBS and fixed for 20 minutes with 4% paraformaldehyde (w/v in HBS) followed by quenching for 10 minutes by incubation with 50 mM NH_4Cl. Coverslips were then rinsed and permeabilized with 0.1% Triton-X-100 for 10

minutes. Dilutions of antibodies used are reported in Table 3. Coverslips were rinsed and blocked for 1 hour with 4% donkey serum. Coverslips were then incubated with mouse anti-HA and rabbit anti-EGFR primary antibodies for one hour, followed by 4 washes for 5 minutes each. Secondary antibodies donkey anti-mouse Alexa Fluor 555 and donkey anti-rabbit Alexa Fluor 647 (Molecular Probes, Eugene, OR) were then added and the coverslips were incubated for 1 hour. Coverslips were then incubated with 1 µg/ml Hoescht 33258 in PBS for one hour to stain nuclei. All antibodies were diluted in 4% donkey serum. Coverslips were washed and mounted on slides in Prolong Gold Antifade mounting medium (Invitrogen) and allowed to dry overnight. After drying, slides were stored at 4^{o}C. Slides were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc.) at the University of Alberta Cell Imaging Facility.

2.9. Statistical Analysis

Statistical analyses were performed using Student's *t*-test, one-way ANOVA or Repeated Measures analysis of variance. All confidence intervals were calculated at a 95% probability level. The statistical calculations were done using Graph Pad Prism 5 software (Prism).

CHAPTER 3: INSIGHTS INTO A ROLE OF LIPINS IN REGULATING PHOSPHOLIPASE D SIGNALLING

3.1. Introduction

The lipins are enzymes that could control the balance between intracellular PA and DAG. We hypothesized that the lipins could attenuate PLD signalling by decreasing PA accumulation and increasing DAG or they could promote other effects of PLD signalling by increasing DAG. The objective of the work in this Chapter was to use genetic approaches including depletion or overexpression of lipin-1 or lipin-2 to determine whether changing lipin expression would cause upregulation or downregulation of PLD signalling and to determine whether the lipins could translocate to membranes in response to treatment of cells with PLD agonists. In order to determine whether the lipins could regulate PLD signalling, we used several human cancer cell lines, Rat2 fibroblasts, and a MEF model. We prepared and characterized *fld* and control MEFs. The *fld* MEFs lack lipin-1 expression and by comparison of PLD signalling in *fld* and control MEFs we have investigated whether lipin-1 is playing a role in attenuating or promoting PLD signalling. The human cancer cell lines were chosen because they showed expression of the lipins as will be demonstrated in this work and because earlier studies showed significant PLD activity in these cell lines [104,105].

We compared the effects of PLD signalling in *fld* and control MEFs as measured by PA and DAG labelling and ERK1/2 activation. We followed up these studies by overexpressing lipin-1 and lipin-2 and measuring PA and DAG labelling to determine whether PLD signalling would be attenuated. We also

examined a role for lipins in translocation to cell membranes in response to PLD activation.

As enzymes that regulate PA/DAG ratios within the cell, lipins could potentially regulate signalling pathways that promote the acquisition of chemoresistance in cancer cells. We therefore characterized PA and DAG accumulation in doxorubicin-resistant (Dox-resistant) and control breast cancer cells. We used chemical inhibitors to estimate the percentage of PA labelling that is due to PLD1 and PLD2 activity in breast cancer cells. Further studies were done in fibroblasts with bromoenol lactone (BEL), a pharmacological inhibitor of lipins, to determine whether lipins could regulate cell migration. We examined the effects of BEL on PLD-dependent cell migration and PA and DAG labelling in Rat2 fibroblasts which have been used previously to study PLD-activated cell migration [131]. Further studies then examined whether knockdown of lipins produced compensatory upregulation of expression of other lipin isoforms.

3.2. Evidence for redundancy of function across lipin isoforms in mouse embryonic fibroblasts

Our first goal was to characterize lipin expression in the MEFs. All three lipin isoforms were expressed in control MEFs and no expression of lipin-1α or lipin-1β was detected in the *fld* MEFs in RT-PCR studies (Fig.3.1A). The *fld* MEFs did not exhibit a significant increase in either lipin-2 or lipin-3 expression

as compared to controls (Fig. 3.1A). Moreover, although there was a trend for decreased Mg^{2+}-dependent PAP activity in *fld* MEFs as compared to controls, it was not significant (Fig 3.1B).

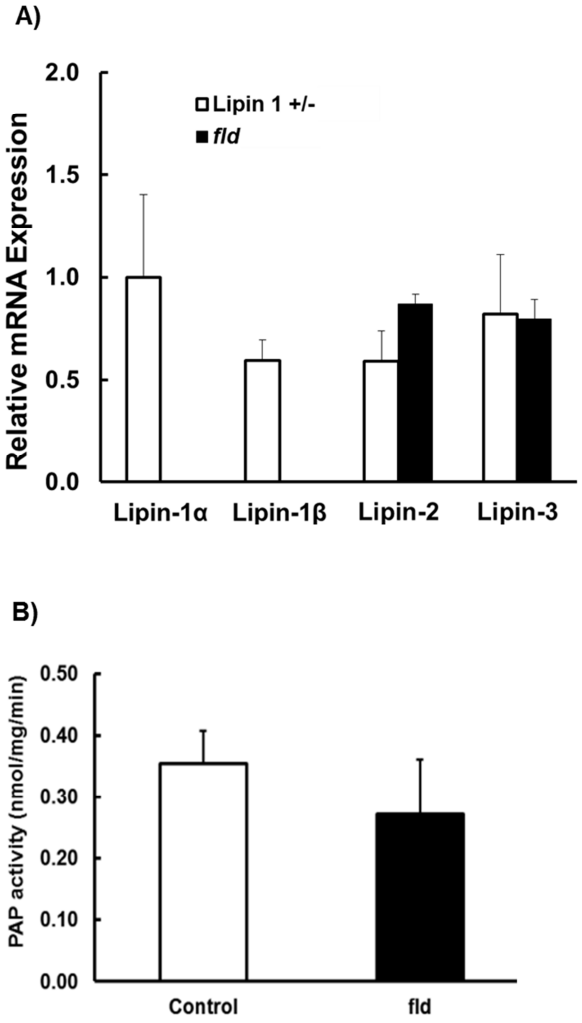


Figure 3.1. Lack of lipin-1 expression in fld MEFs is not associated with significant compensation in mRNA expression of lipin-2 and lipin-3 isoforms despite the high PAP activity in *fld* MEFs. A. Relative mRNA levels of each lipin isoform are shown in comparison to the mRNA level of lipin-1A, which is set at a value of 1 in the control. The lipin mRNA levels were normalized to mRNA levels of cyclophilin A. Values represent means ± S.E.M. from 6 independent observations per treatment. The means were not significantly different. **B. The Mg^{2+}-dependent PAP activity in *fld* MEFs did not differ from activity in control MEFs.** The results represent means ± S.E.M. from 5 to 6 independent observations per treatment. The means were not significantly different.

3.3. Knockdown of lipins does not cause a compensatory increase in expression of other lipin isoforms

Consistent with our results on the effect of lipin-1 depletion in *fld* and control MEFs, we found that knockdown of lipins in MDA MB 231 cells failed to show reciprocal upregulation of the relative mRNA expression of the other isoforms. We chose to use MDA MB 231 breast cancer cells as our cell system for knockdowns because siRNA-mediated knockdown can be more easily achieved in cancer cells and because these cells and many other breast cancer cell lines express high levels of PLD [104] and would be suitable for future experiments to investigate effects of lipin-2 or lipin-3 depletion on PLD signalling. PLD signalling including increased SK-1 translocation to membranes is also reported in breast cancer cell lines. A significant knockdown of each isoform individually was achieved at $p<0.05$ (Fig. 3.2A-C). This is the first time that lipin-3 knockdown has been reported (Fig. 3.2C).

Knockdown of lipin-1 did not affect the expression levels of lipin-2 or lipin-3 (Fig 3.2A). In contrast to studies which reported reciprocal upregulation of lipin-2 when lipin-1 was knocked down and vice versa [173], upregulation of the other lipin isoforms when one isoform was depleted was not observed (Fig.3.2C). Knockdown of all three of the lipin isoforms were done individually and in a triple knockdown with siRNA (Fig.3.2A-C). However, we were unable to achieve a significant triple knockdown of the lipins except with siRNA for lipin-2 (Fig. 3.2A-C). However, because of the redundancy of PAP activity we observed in MEFs (Fig. 3.1B), it is likely that knockdown of individual isoforms might not be

sufficient in order to achieve a knockdown of expression that will allow us to characterize the role of lipins in PLD-mediated signalling through PA or to attenuate the proposed effects of regulation of PA levels by the lipins.

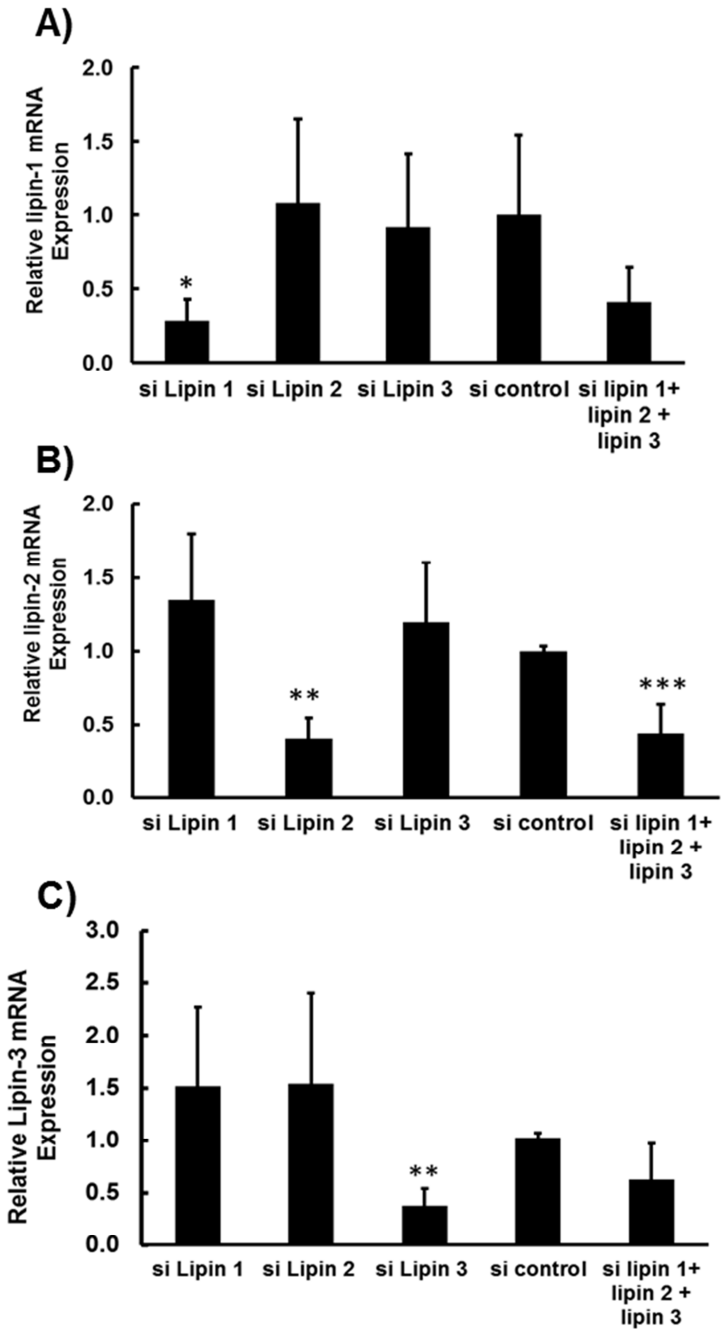


Figure 3.2. Lack of compensation of expression of lipin isoforms in MDA MB 231 breast cancer cells in which lipin isforms have been knocked down with siRNA. Relative mRNA levels of each lipin isoform are shown in comparison to the mRNA level of lipin-1 which is set at a value of 1 for lipin-1. The lipin-1, lipin-2 and lipin-3 mRNA levels were normalized to mRNA levels of cyclophilin A. Results represent means ± S.E.M. from at least 3 independent experiments. Significance is as follows: *, si-lipin sample different from si-control (p<0.05), **, si-lipin sample different from si-control (p<0.0001), ***, si-lipin sample different from si-control (p<0.005)

3.4. Determination of phosphatidylcholine levels and phospholipase D activity in control and *fatty liver dystrophy* mouse embryonic fibroblasts

In order to confirm that there are not differences in labelling in PC between *fld* and control MEFs, which would affect PLD signalling, we measured labelling in PC (Fig. 3.3A). There were no significant differences in basal or endothelin-1-stimulated levels of PC labeling between *fld* and control MEFs (Fig. 3.4A). PC hydrolysis was also not affected by depletion of lipin-1 (Fig. 3.3A). To test the possibility that *fld* and control MEFs could have differential PLD activity, we also measured PLD activity as stimulated by endothelin-1 or LPA in *fld* and control MEFs. Figs. 3.3B-C represent preliminary experiments and show that there are not very large differences between basal and stimulated levels of PLD activity in the *fld* as compared to the control MEFs, although the significance cannot be calculated because the values represent means with ranges. In addition, there is no consistent trend for increased PLD activity in *fld* or control MEFs as stimulated by LPA or endothelin-1 (Fig. 3.3B-C). A larger sample size would be needed to determine significance, which is not possible from the means ± ranges shown in Fig. 3.3B-C.

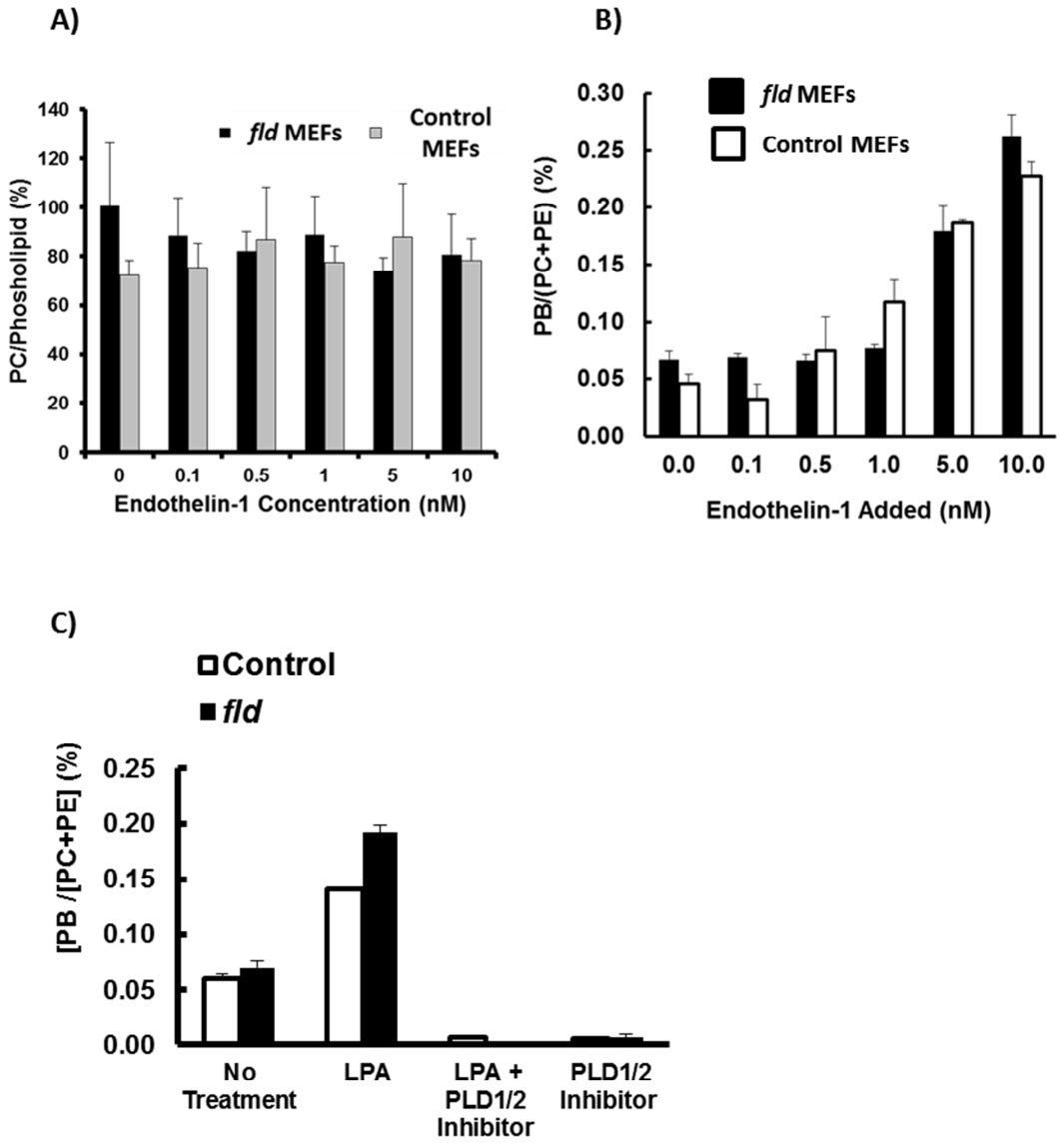


Figure 3.3. Preliminary evidence for no differences in PC labelling or PLD activation in *fld* or control MEFs. A. Labelling of PC in *fld* and control MEFs. Results are means ± S.E.M. from at least 3 independent observations per treatment. The means were not significantly different. **B. PLD activity in *fld* and control MEFs as stimulated by LPA.** Cells were treated with 0.5 μM LPA for 2 min. **C. PLD activation in response to endothelin-1 in *fld* and control MEFs.** For B and C, means ± ranges are shown from two experiments.

3.5. Depletion of lipin-1 does not affect phospholipase D-mediated phosphatidic acid accumulation or diacylglycerol levels in mouse embryonic fibroblasts in labelling experiments

Labelling studies were done in MEFs prepared from *fld* mice. Endothelin-1 stimulated the production of PA in a dose-dependent manner (Fig. 3.4A). A significant increase in labelling in PA in both *fld* and control MEFs in response to endothelin-1 at $p<0.001$ was observed at 1 nM endothelin-1 and peaked at 5 nM of endothelin-1 (Fig. 3.4A). This increase was sensitive both to PLD1 and PLD2 inhibition with pharmacological inhibitors. In contrast, DAG levels did not increase significantly in response to endothelin-1 treatment (Fig. 3.4B). It was hypothesized that decreased DAG production should be observed in *fld* MEFs as compared to controls due to the lack of the PAP activity of lipin-1 to convert PA to DAG. However, the magnitude of the difference in DAG levels between *fld* MEFs compared to control MEFs was not significant (Fig. 3.4B). The time of endothelin-1 treatment for optimal PLD activation was determined previously in MEFs and Rat2 fibroblasts to be 5 min [131] and verified in experiments demonstrating translocation of PAP activity (Fig. 3.4A). However, there was no significant difference between *fld* and control MEFs in PA accumulation in response to PLD stimulation by growth factors (Fig. 3.4A). There were no significant differences in basal levels or agonist-stimulated levels of PA in *fld* as compared to control MEFs (Fig. 3.4A-B). We concluded that treatment with endothelin-1 did not cause a difference in PA accumulation between *fld* and control MEFs at a statistically significant level and PA levels were increased in both *fld* and control MEFs.

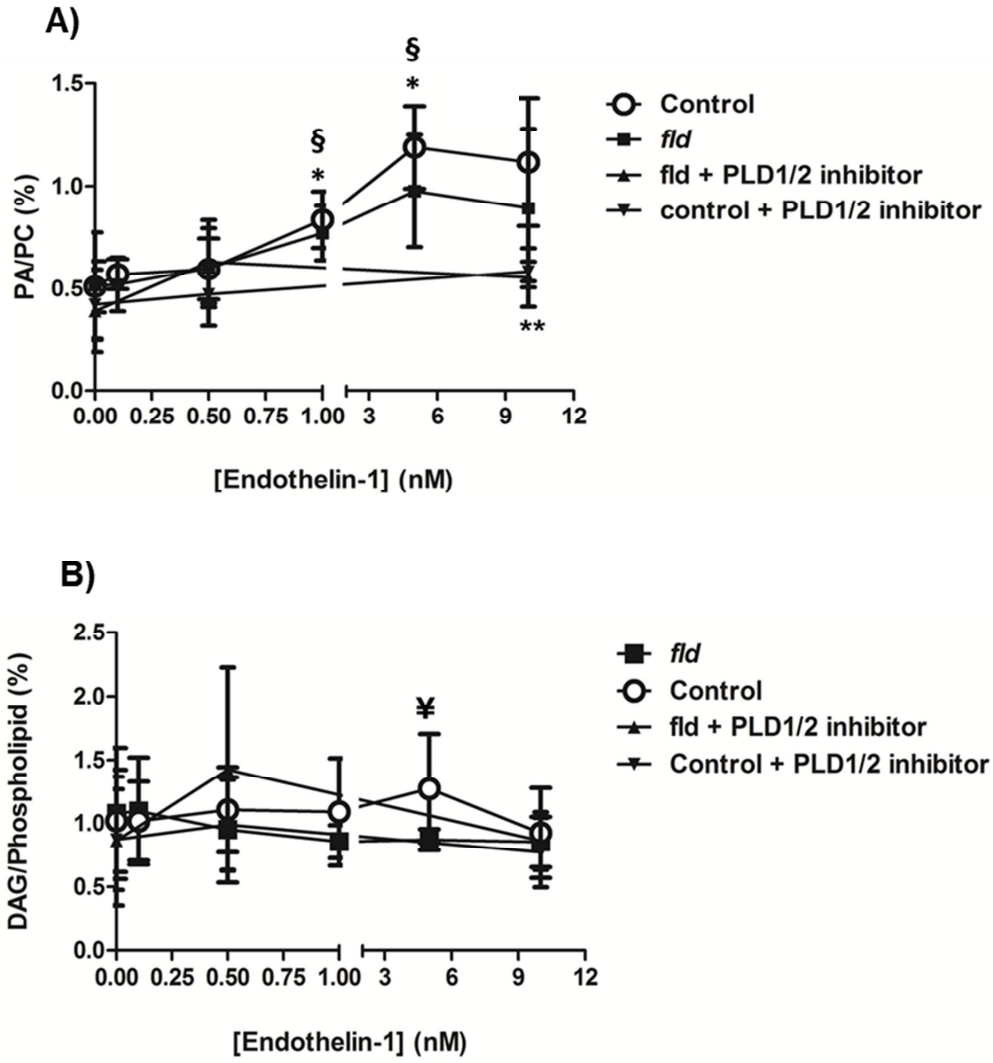


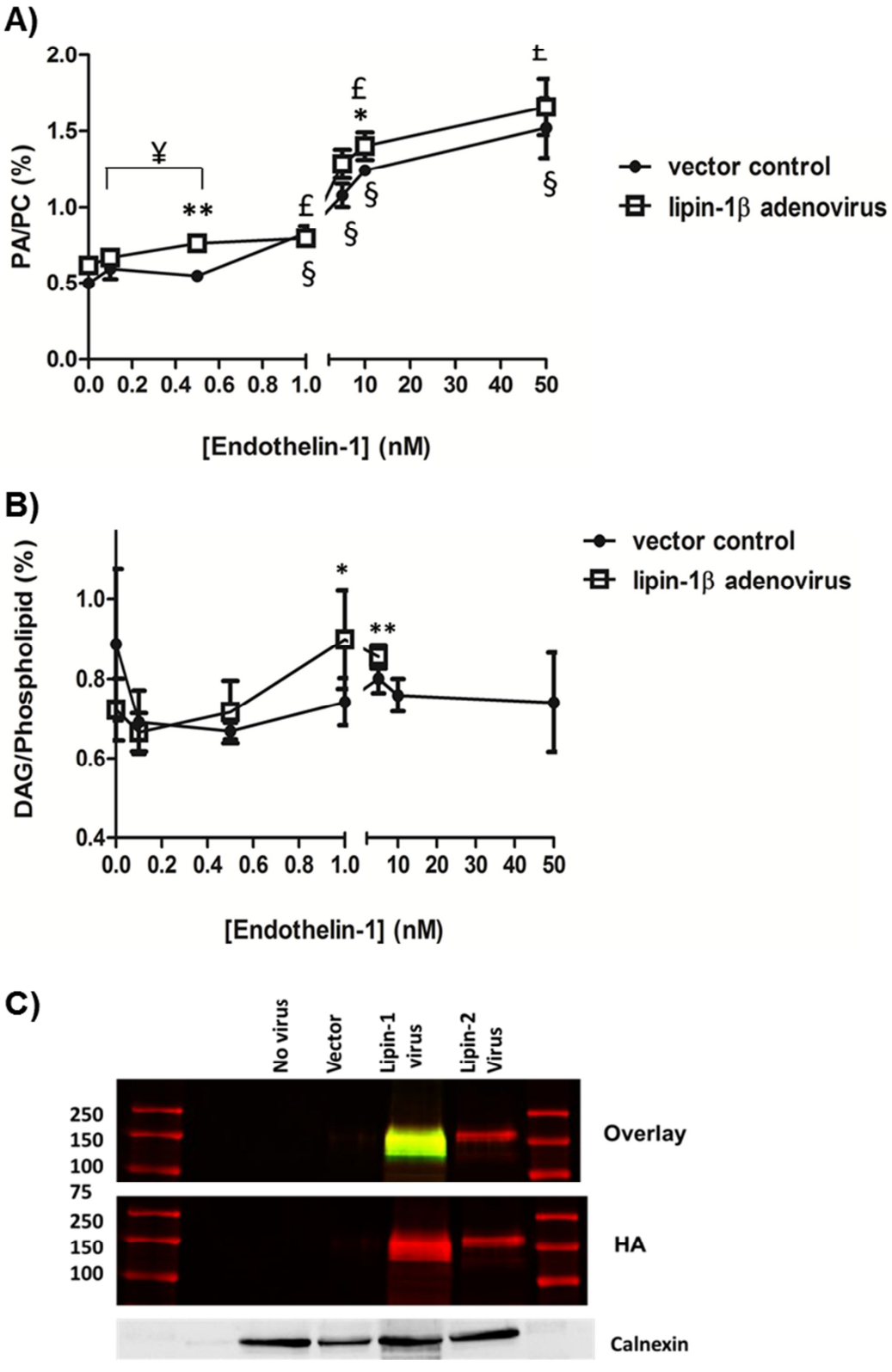
Figure 3.4. Endothelin-1 treatment causes an increase in labelling in PA, which is abrogated by a pan PLD inhibitor. Cells were prelabelled with [^{3}H]-palmitic acid for 2 hours followed by endothelin-1 treatment. **A. Endothelin-1 causes increased PA accumulation in both *fld* and control MEFs. B. Endothelin-1 does not cause increased labelling in DAG except for the 5 nM endothelin-1 treatment for which there was a significant difference in DAG levels between *fld* and controls. Control and *fld* MEFs were treated with 50 nM endothelin-1 for 5 min.** Values represent means ± S.E.M. from 6 independent observations for treatments with VU0155056, a dual PLD1/2 chemical inhibitor and 9 to 15 independent observations without PLD inhibitor treatment. *, different from untreated control (p<0.05), §, control MEFs are different from untreated control (p<0.0001), **, control group different from group without inhibitor (p<0.01).

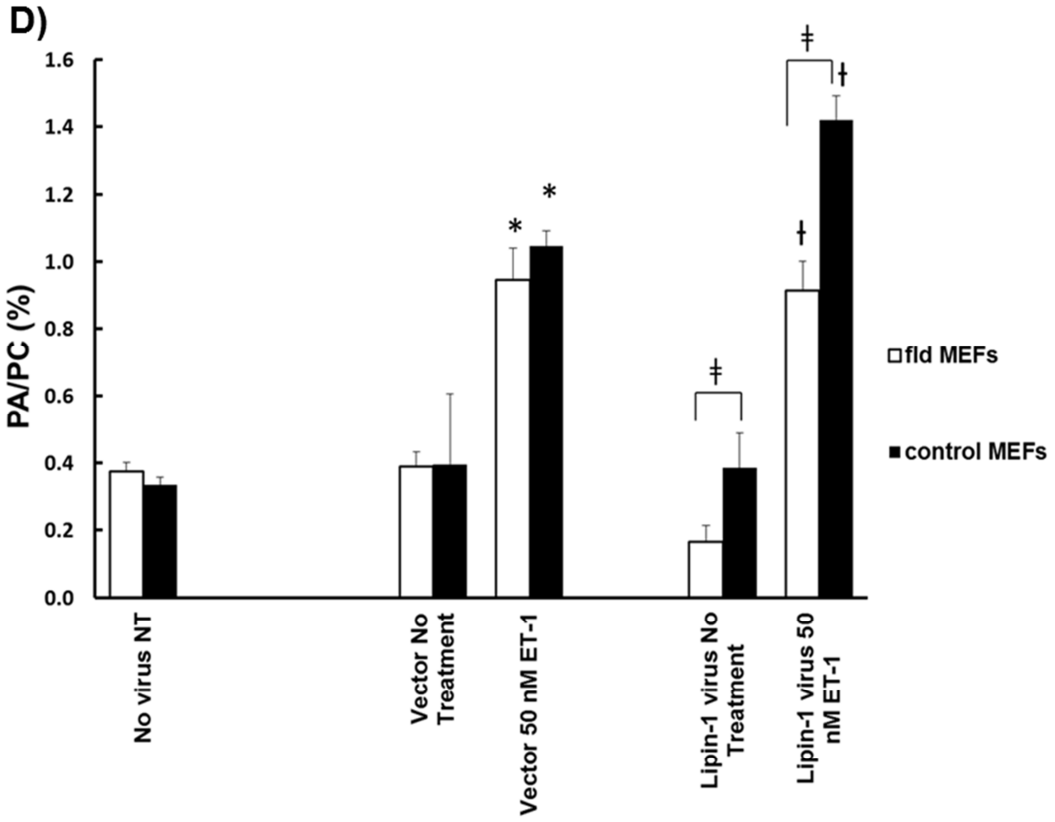
3.6. Overexpression of lipin-1 does not affect phospholipase D-mediated phosphatidic acid accumulation in mouse embryonic fibroblasts

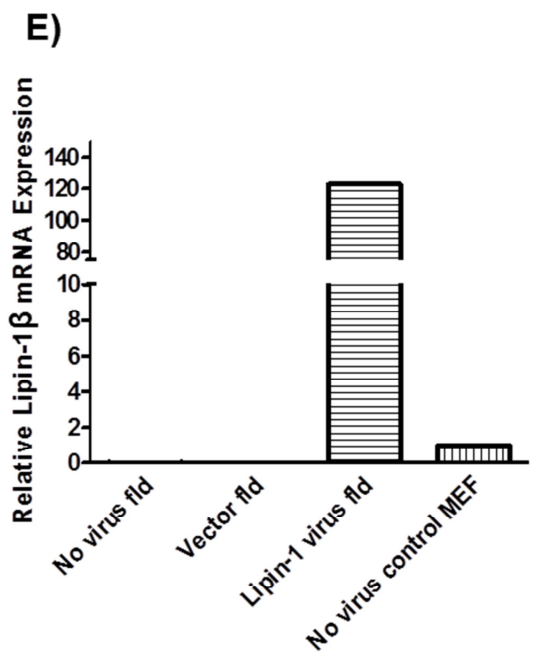
Labelling experiments were done in MEFs overexpressing lipin-1. Radioactivity in PA was quantified and expressed as a percentage of the total lipid formation. Overexpression of lipin-1 or lipin-2 did not abrogate the PA accumulation in response to PLD activation (Fig 3.5A-E). PA accumulation was significant as compared to the untreated control (Fig.3.5A). There was no difference in basal levels of PA between the vector control and the lipin-1 overexpressing MEFs (Fig. 3.5A-B). However, after 5 min. of stimulation with 1 nM of endothelin-1, there was a significant increase in DAG levels in the lipin-1 overexpressing MEFs as compared to the controls at $p<0.05$ (Fig. 3.5B). In addition, overexpression of the lipin-1β isoform by transfection of *fld* MEFs with an adenoviral vector did not result in a decrease in endothelin-1-stimulated PA accumulation (Fig. 3.5A). With a larger sample size, however, we might expect to see more significant differences between vector-infected and lipin-1 overexpressing *fld* MEFs in Fig. 3.5A.

Consistently, we found that only at 4 min of LPA treatment did *fld* MEFs overexpressing lipin-2 show a significant increase in DAG accumulation compared to *fld* MEFs infected with the vector control adenovirus (Fig. 3.5F). Since DAG levels have been found to remain unchanged in response to LPA treatment over a treatment course of minutes in previous work [131], this result where only treatment point was significant indicated that there is no effect of overexpression of lipin-2 on DAG levels in MEFs (Fig. 3.5F). In the same

experiments, we confirmed the overexpression of lipin-1 and lipin-2 through RT-PCR analysis of lipin-1 mRNA expression (Fig. 3.5E) and through western blot analysis (Fig. 3.5C). PA accumulation in response to stimulation with endothelin-1 was greater in control MEFs than in *fld* MEFs, which were infected with HA-tagged lipin-1β adenovirus (Fig. 3.5D). Interestingly, we observed a significant increase in the PA level in control MEFs compared to *fld* MEFs, both in the presence of or without PLD activation through endothelin-1 treatment (Fig. 3.5D). However, in *fld* MEFs, the basal levels of lipin-2 and lipin-3 are able to compensate for the loss of PAP activity resulting from the absence of lipin-1 expression (Fig. 3.5A-B). From this analysis it was concluded that overexpression of lipin-1 or lipin-2 in *fld* MEFs does not affect bulk cellular DAG levels (Fig. 3.5A-B).


A)
PA/PC (%)
2.0
1.5
1.0
0.5
0.0
0.0 0.2 0.4 0.6 0.8 1.0 10 20 30 40 50
[Endothelin-1] (nM)
¥
**
£
*
§
£
§
§
£
vector control
lipin-1β adenovirus
B)
DAG/Phospholipid (%)
1.0
0.8
0.6
0.4
0.0 0.2 0.4 0.6 0.8 1.0 10 20 30 40 50
[Endothelin-1] (nM)
*
**
vector control
lipin-1β adenovirus
C)
No virus
Vector
Lipin-1 virus
Lipin-2 Virus
250
150
100
75
250
150
100
Overlay
HA
Calnexin


D)
PA/PC (%)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
fld MEFs
control MEFs
No virus NT
Vector No Treatment
Vector 50 nM ET-1
Lipin-1 virus No Treatment
Lipin-1 virus 50 nM ET-1


E)
Relative Lipin-1β mRNA Expression
140
120
100
80
10
8
6
4
2
0
No virus fld
Vector fld
Lipin-1 virus fld
No virus control MEF

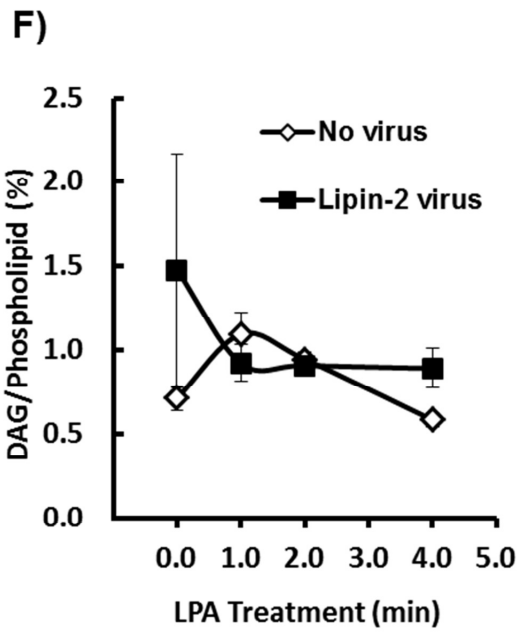
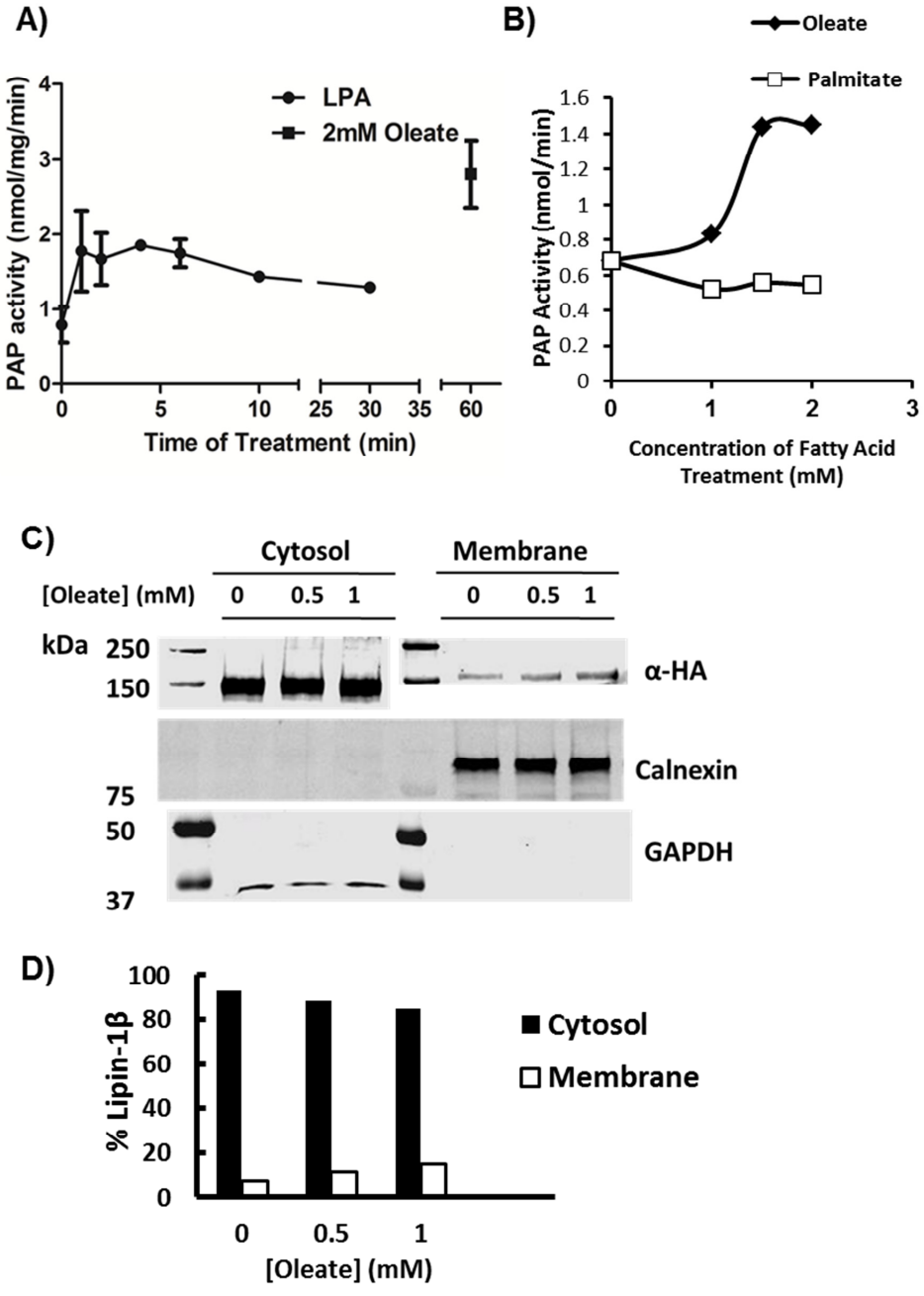

F)
DAG/Phospholipid (%)
2.5
2.0
1.5
1.0
0.5
0.0
No virus
Lipin-2 virus
0.0 1.0 2.0 3.0 4.0 5.0
LPA Treatment (min)

Figure 3.5. PA labelling in fld MEFs and in MEFs overexpressing lipin-1. Samples were treated with endothelin-1 for 5 min. **A. PA labelling in MEFs infected with vector control or adenovirus for lipin-1β.** For A, values represent means ± SEM from 3 independent experiments, except for the adenovirus-infected samples which were not treated with endothelin-1 which represent means ± ranges. Significance is indicated as follows: *, lipin-1 overexpressing different from empty vector (p<0.05), **, lipin-1 overexpressing different from vector (p<0.002), §, vector-infected samples different from untreated control, (p<0.001), ¥, lipin-1 overexpressing samples different from lipin-1 overexpressing sample treated with 0.1nM endothelin-1 (p<0.05), £, lipin-1 overexpressing samples different from lipin-1 overexpressing sample treated with 0.1 nM endothelin-1 (p<0.01). **B. DAG labelling in MEFs infected with vector control or adenovirus for lipin-1β.** Significance is as follows: For lipin-1 overexpressing samples, *, different from 0.1 nM endothelin-1-treated sample (p<0.05), **, different from 0.1 nM endothelin-1-treated sample (p<0.01). **C. Overexpression of lipin-1 and lipin-2 in fibroblasts as quantified by a representative western blot from two independent experiments.** Blots were incubated with antibodies against lipin-1 (observed in the green channel) and against the HA tag (observed in the red channel). The two channels were superimposed and the yellow regions of the blot represent the superposition of the HA antibody and the lipin-1 antibody. Overexpression of lipin-1 in *fld* and control MEFs as quantified by RT-PCR. **D. Comparison of effect of lipin-1 overexpression on PA labelling in *fld* and control MEFs.** Values represent means ± S.E.M. from 5 independent observations per treatment. NT stands for No treatment. Significance is indicated as follows: ǂ, p<0.05, *, significantly different from no treatment vector-infected control (p<0.01), ł, significantly different from lipin-1 adenovirus-infected no treatment control (p<0.01). **E. Overexpression of lipin-1 in *fld* and control MEFs as quantified by RT-PCR. F. Overexpression of lipin-2 caused significantly increase in DAG labelling as compared to the no virus control.** Values represent means ± S.E.M. for 3 independent observations per treatment.

3.7. Increased PAP activity is associated with membranes in response to stimulation with phospholipase D agonists in cancer cells

Endothelin-1 stimulates translocation of endogenous PAP activity to membranes in the HT1080 human fibrosarcoma cell line (Fig. 3.6E,G) and LPA exerts a similar effect (Fig. 3.6F). Oleate, which is known to stimulate a high level of PAP translocation, was used as a positive control for translocation (Fig. 3.6G). Translocation of the lipin-1 isoform was confirmed by western blot analysis from one blot using the same membrane fraction samples as in Fig. 3.6G, which showed that endogenous lipin-1 translocates to membranes as detected with an antibody specific to the C-terminus of lipin-1 (Fig. 3.6G). In Fig. 3.6G, there is a time trend for translocation of PAP activity based on the means ± ranges. There is an obvious overall time dependency trend that peaks at 2 min of stimulation with LPA and forms a plateau until 10 min. of stimulation (Fig. 3.6G). Endothelin-1 stimulation consistently caused increased PAP activity in the membrane fraction, which peaks at 6 min. and then there is a trend for a decline (Fig.3.6E,G). However, statistical significance could not be determined because values represent means ± ranges (Fig. 3.6E,G). The translocation of PAP activity to membranes was approximately two-fold in response to both LPA or endothelin-1, which was not as high as the translocation of oleate (Fig. 3.6A-D,H). Future work with larger sample sizes and more experiments is needed to determine whether the translocation is significant and the time dependency of translocation. We also showed that all three lipin isoforms are expressed in HT1080 cells through measurement of mRNA levels by RT-PCR (Fig. 3.6F). RT-

PCR analysis detected fairly high expression of lipin-1 and lipin-2 isoforms and lipin-3 mRNA expression was also detected in HT1080 cells in one experiment (Fig. 3.6F) and later RT-PCR experiments confirmed expression of all three lipin isoforms in MDA MB 231 cells (Fig. 3.12A-C).



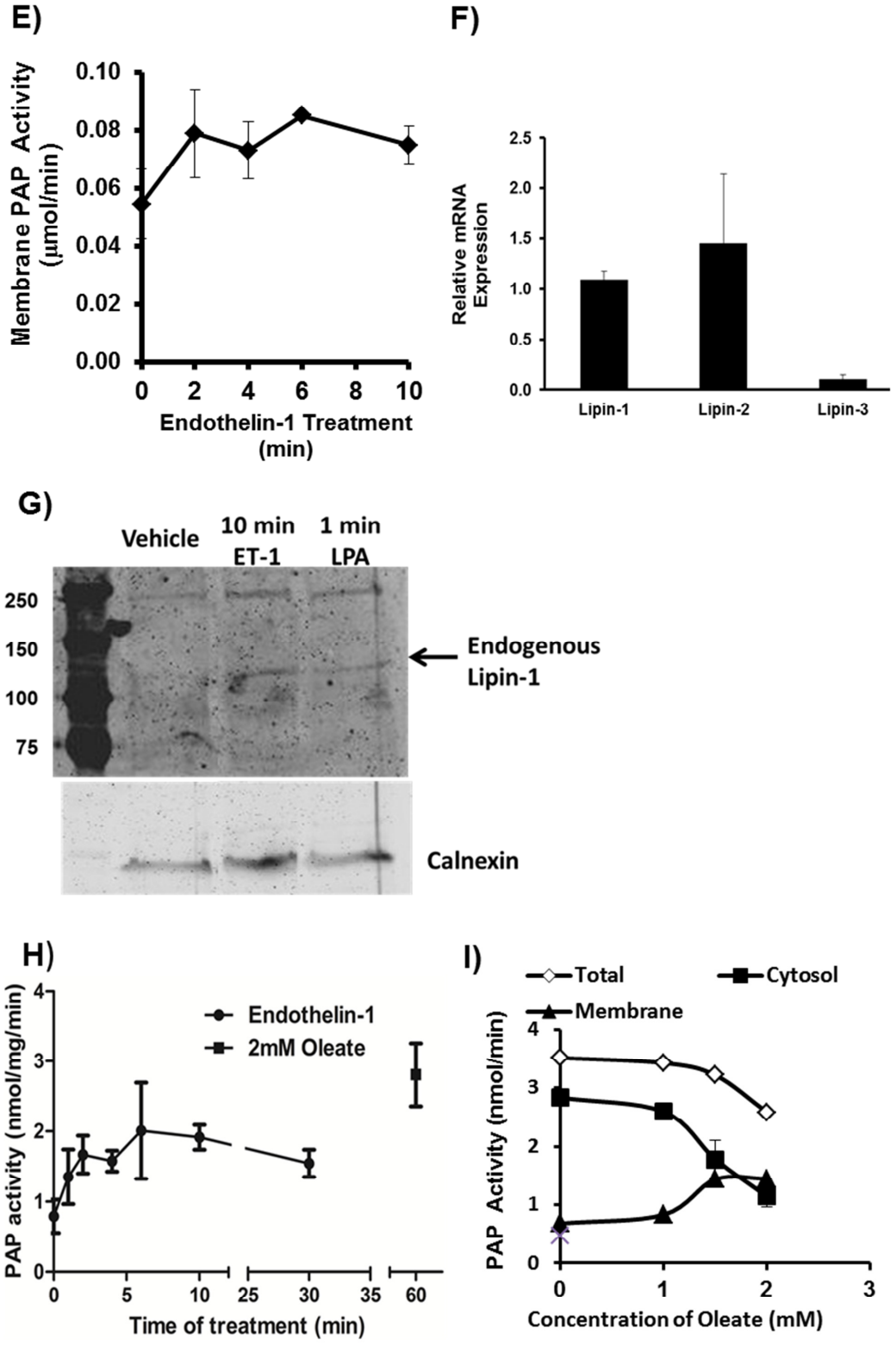

E)
Membrane PAP Activity (µmol/min)
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
Endothelin-1 Treatment (min)
F)
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
Lipin-1
Lipin-2
Lipin-3
G)
Vehicle
10 min ET-1
1 min LPA
250
150
100
75
Endogenous Lipin-1
Calnexin
H)
PAP activity (nmol/mg/min)
Endothelin-1
2mM Oleate
0
5
10
25
30
35
60
Time of treatment (min)
I)
Total
Cytosol
Membrane
PAP Activity (nmol/min)
Concentration of Oleate (mM)

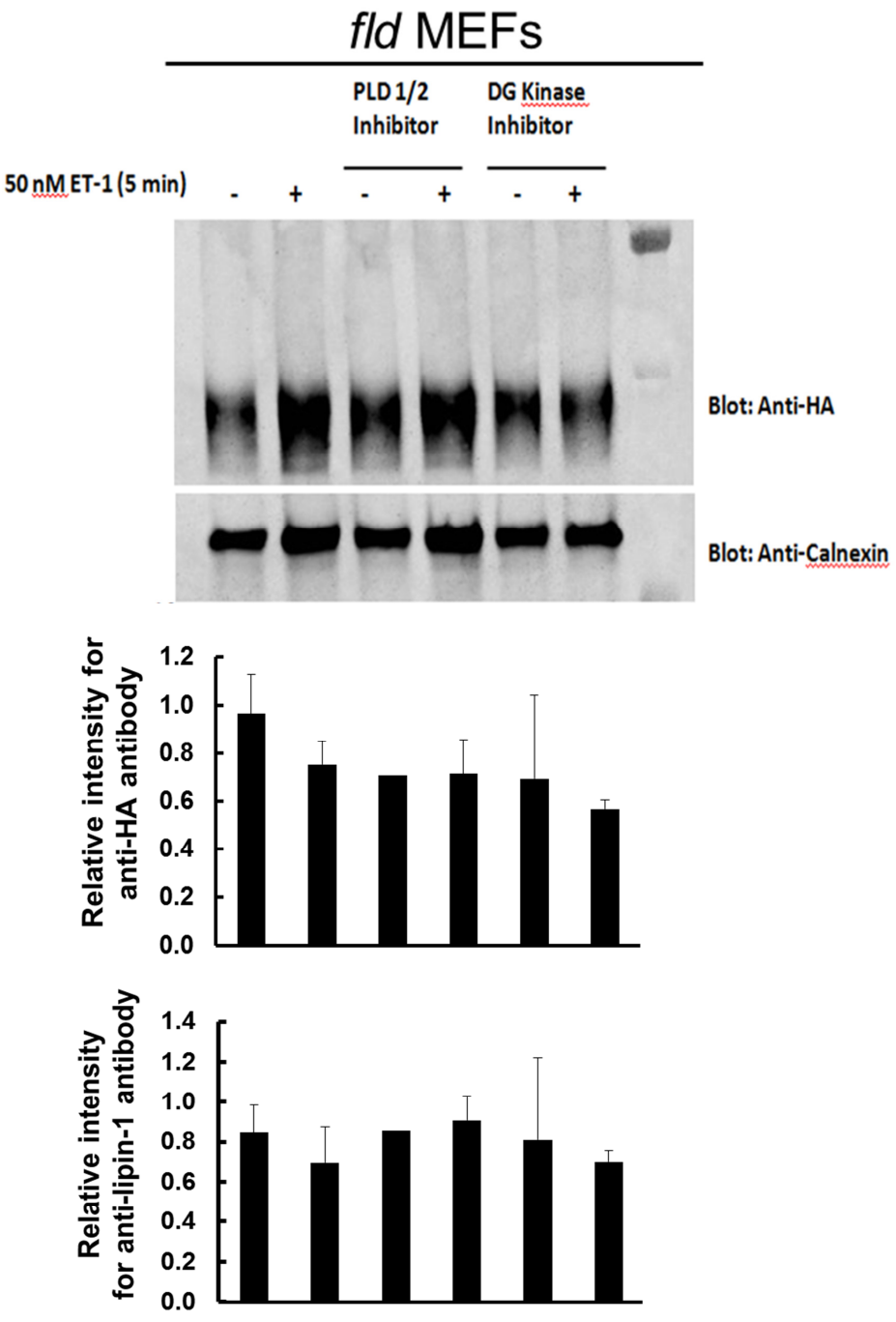
Figure 3.6. PAP activity shows increased association with membranes in response to stimulation with PLD agonists in HT1080 and MDA MB 231 cells. Oleate, which is known to stimulate translocation of PAP activity to membranes, is used as a positive control for translocation. Membrane fractions were prepared by digitonin lysis. **A. Association of PAP activity with membranes of HT1080 cells increases over time in response to treatment with 0.5 μM LPA for the indicated time course or treatment with 2 mM oleate for 60 min. B. PAP activity of membranes induced with dexamethasone for 17 hours and lysed by digitonin. C. Lipin-1β was overexpressed in rat hepatocytes and cells were lysed by digitonin to separate the membrane and cytosolic fractions.** Each fraction was analyzed by western blotting with antibodies against the HA tag. Calnexin was used as the loading control for the membrane fraction and GAPDH was used as the loading control for the cytosolic fraction. **D. Quantification of western blot from Panel C.** The signal from the anti-HA antibody was normalized to calnexin as a loading control. **E. Association of PAP activity with membranes in MDA MB 231 cells infected with lipin-1β adenovirus.** Cells were treated with 50 nM of endothelin-1. Values represent means ± ranges from 2 independent observations. **F. Expression of all three lipin isoforms is found in HT1080 cells as determined from RT PCR from one experiment.** Relative mRNA levels of each lipin isoform are shown in comparison to the mRNA level of lipin-1A, which is set at a value of 1 in the Rat2 fibroblast control. The lipin mRNA levels of lipin-1 and lipin-3 were normalized to mRNA levels of cyclophilin A. **G. Translocation of lipin-1 in HT1080 cells treated with 50 nM endothelin-1 or 0.5 μM LPA for the indicated times.** Western blot analysis of endogenous lipin-1 translocation from the same samples in which PAP activity was measured is shown and is from one experiment. The western blot was analyzed for lipin-1 expression with an antibody specific to the C-terminus of lipin-1and probed for calnexin as a control. **H. Increased association of PAP activity with membranes from HT1080 cells treated with 50 nM endothelin-1 for the indicated time course or treated with 2 mM oleate for 60 min. I. PAP activity of rat hepatocytes induced with dexamethasone for 17 hours and treated with fatty acid for 1 hour followed by digitonin lysis.**

3.8. Recombinant lipin-1 does not translocate to membranes in response to stimulation with phospholipase D agonists

We hypothesized that lipins should translocate to the membranes where they could dephosphorylate PA that accumulates in response to PLD activation with endothelin-1 as an agonist. We treated MDA MB231 cells, which are a breast cancer cell line expressing endothelin-1A and endothelin-1B receptors [108], with endothelin-1 to activate PLD-dependent signalling. However, lipin-1 translocation in response to PLD activation did not occur to a significant extent (Fig. 3.7A-B). The PLD agonist endothelin-1 does not stimulate translocation of recombinant lipin-1β to membranes in *fld* MEFs and PLD inhibitors and a DAG kinase inhibitor did not affect translocation of recombinant lipin-1β (Fig. 3.7A-B).

There was no significant difference with treatment with endothelin-1 caused an approximately 1.6-fold increase in membrane-associated lipin, which occurred consistently (Fig. 3.7). This effect occurred with overexpressed lipin-1 in *fld* MEFs as determined by western blot analysis (Fig. 3.7A) whereas PAP activity translocated to membranes in MDA MB 231 cells (Fig. 3.6A,E,G-H). Treatment with a pan-PLD inhibitor did not affect lipin-1 translocation (Fig. 3.7A). We observed that over-expressed lipin-1 is already present on the membrane in *fld* MEFs (Fig. 3.7A-B) which could raise the background levels for detection of translocation to membranes by western blotting analysis methods. However, these experiments need to be repeated with larger sample sizes.

A)


fld MEFs
PLD 1/2 Inhibitor
DG Kinase Inhibitor
50 nM ET-1 (5 min)
- + - + - +
Blot: Anti-HA
Blot: Anti-Calnexin
Relative intensity for anti-HA antibody
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative intensity for anti-lipin-1 antibody
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

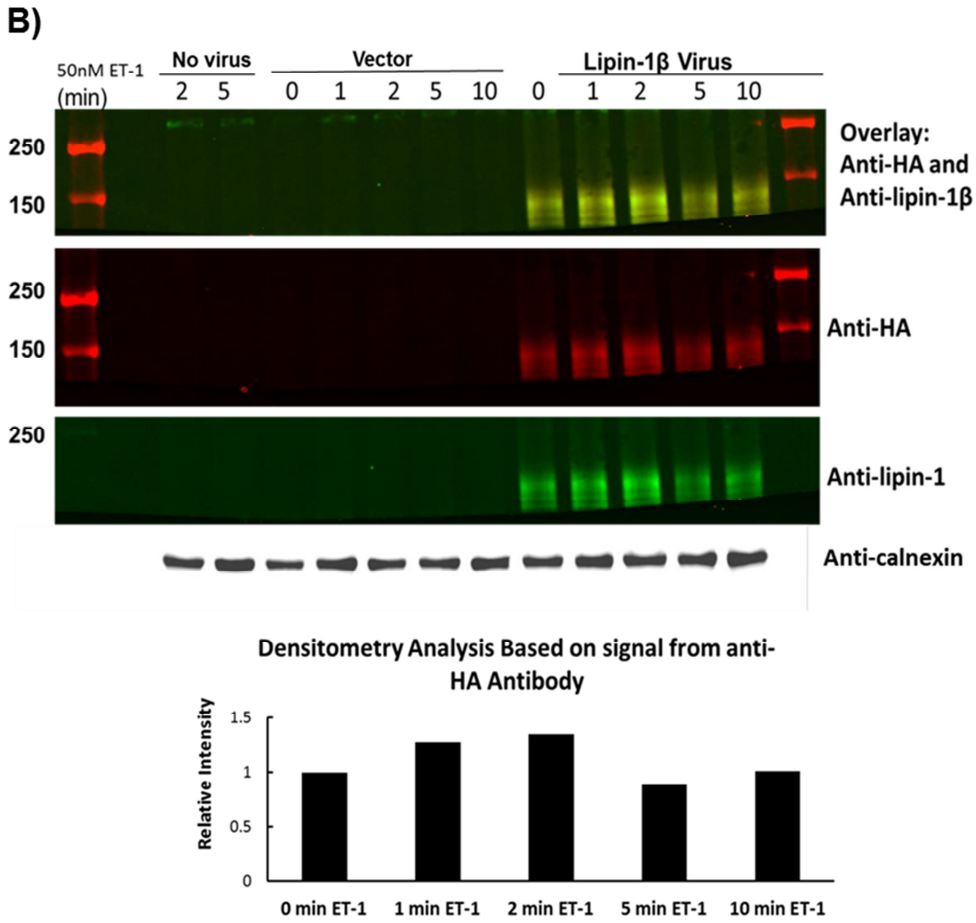


Figure 3.7. Lipin-1 shows a small translocation to membranes and PLD or DGK inhibitors partially counteract this effect. MEFs from *fld* mice were pre-incubated with vehicle or inhibitor for 30 min. followed by treatment with 50 nM endothelin-1 for the indicated time. MEFs were then lysed with digitonin and the membrane fraction was collected and analyzed by western blotting. **A. MEFs from *fld* mice were infected with adenovirus for HA-lipin-1β.** A representative western blot showing translocation of lipin-1β overexpressed in *fld* MEFs. HA-lipin-1 was detected with an anti-HA antibody. Bands were quantified by densitometry and normalized to calnexin expression. Values represent means ± ranges except for the no treatment and endothelin-1-only treated samples which represent means ± S.D. and the sample which received PLD1/2 inhibitor only was from one experiment only. **B. Time course of lipin-1 translocation to membranes in *fld* MEFs from one experiment.** Cells were either not infected or infected with vector control adenovirus or adenovirus for HA-lipin-1β. HA-lipin-1β was detected with an anti-HA antibody (red signal) and with a lipin-1 C-terminus-specific antibody (green signal) and the superposition of these two signals is indicated in the yellow bands. The densitometry represents a quantification of the lipin-1β overexpressing samples of the western blot and lipin-1 expression was normalized to calnexin expression. Values are from one experiment.

3.9. Preliminary evidence that lipin-1 overexpression in *fld* mouse embryonic fibroblasts does not affect ERK1/2 activation although phospho-ERK1/2 translocates to membranes in response to phospholipase D activation

To test for a possible role of lipin-1 in promoting or inhibiting ERK1/2, western blotting analysis was performed on membrane fractions from *fld* MEFs that were either not infected, infected with a vector control, or infected with adenovirus encoding for lipin-1β. The *fld* MEFs were treated with endothelin-1 for 5 min. Western blot analysis detected two bands at 42 and 44 kDa for each of ERK1/2 and pERK1/2, respectively (Fig. 3.8). Treatment of *fld* MEFs with endothelin-1 appears to equally activate ERK1/2 in non-infected cells, cells infected with adenoviral vector, and cells overexpressing lipin-1β. Basal levels of ERK activation between *fld* MEFs and *fld* MEFs overexpressing lipin-1 also do not appear to differ (Fig. 3.8). Preliminary evidence from the western analysis from one experiment showed there was no difference in phosphorylation of ERK1/2 at the membrane in response to endothelin-1 treatment in non-infected control compared to the vector-infected control and lipin-1 overexpressing cells (Fig. 3.8). However, endothelin-1 did stimulate the translocation of p-ERK1/2 to the membranes (Fig. 3.8).

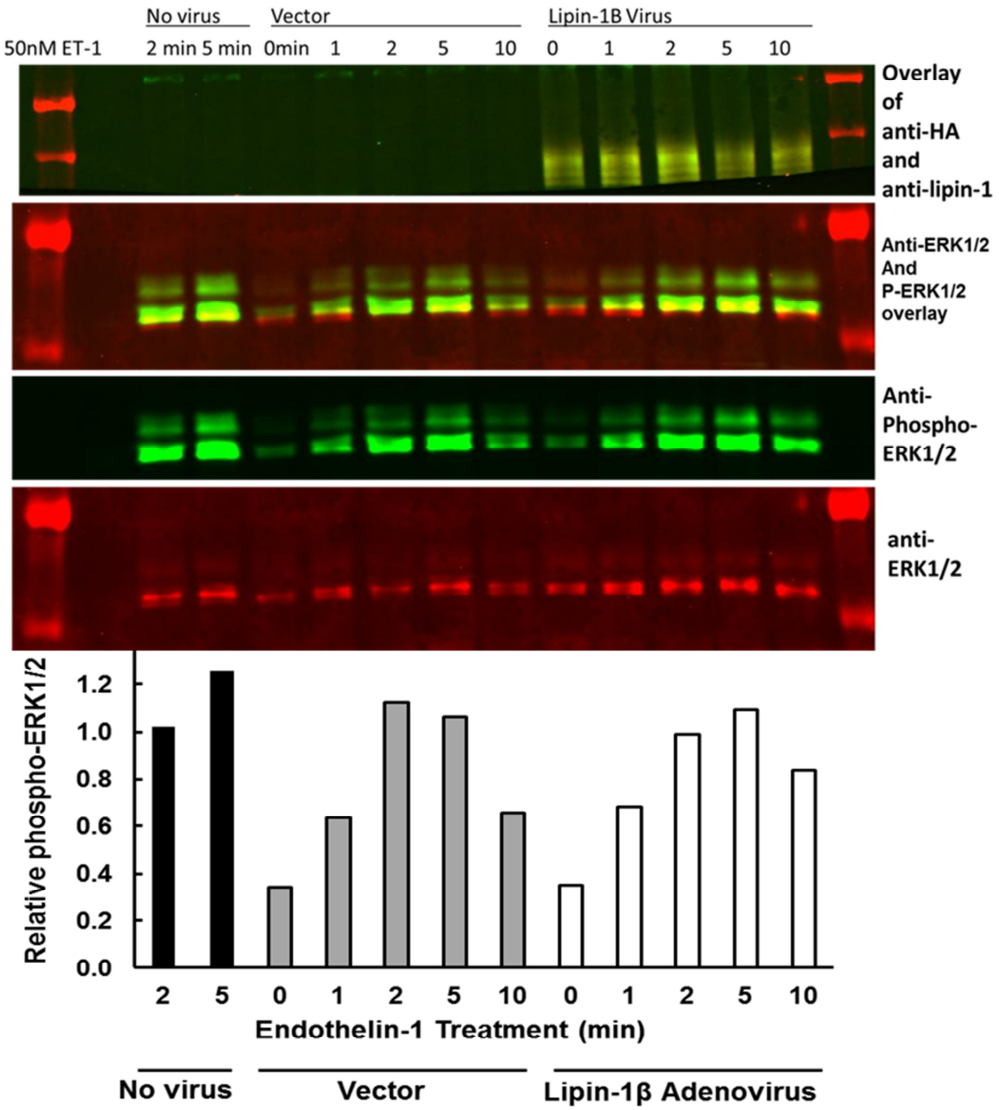
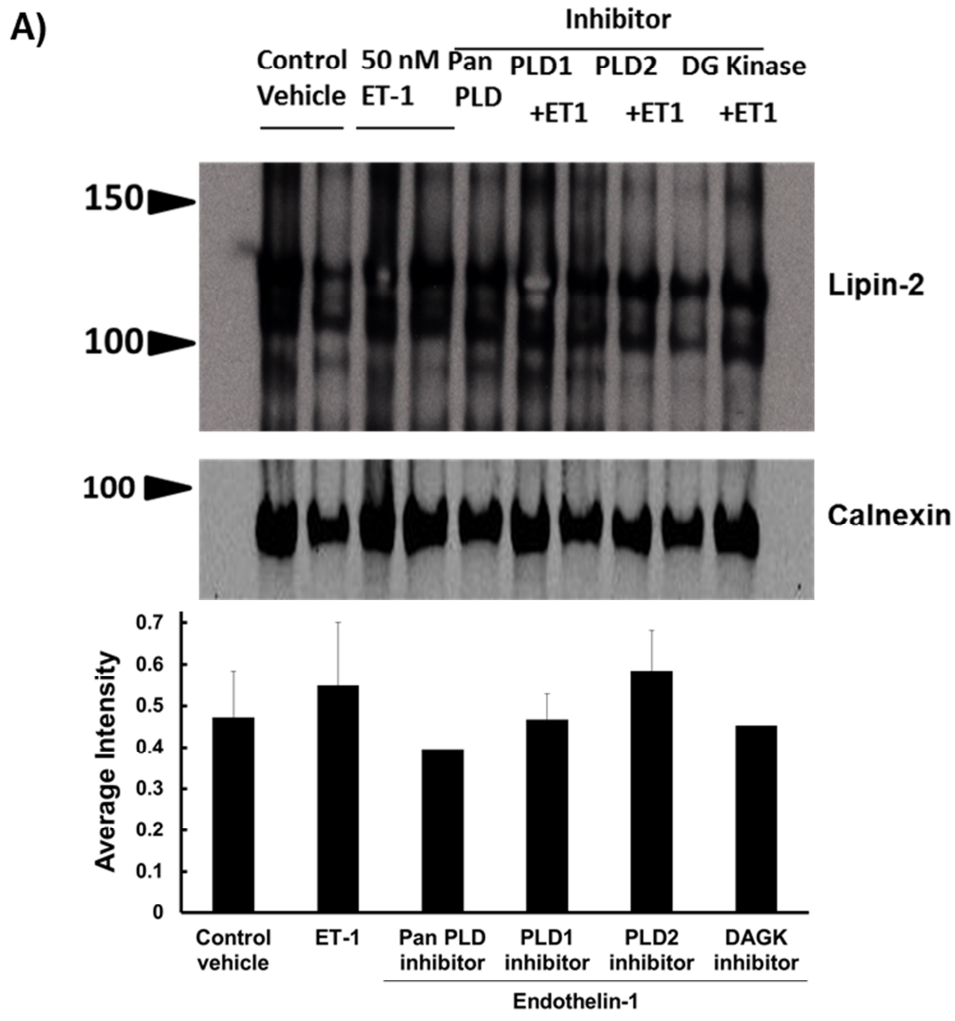


Figure 3.8. Overexpression of lipin-1β in MEFs does not affect the phosphorylation of ERK1/2 as compared to vector or non-infected controls. Western blot analysis of lipin-1, ERK1/2, and pERK1/2, in the membrane fraction collected from digitonin lysis of *fld* MEFs. The cells were either not infected (black bars) or infected with adenovirus for vector control (grey bars) or adenovirus for HA-lipin-1β (white bars) without infection by adenovirus or overexpressing vector or lipin-1. The cells were treated with 50 nM of endothelin-1 for the time indicated. Western blot is from one experiment and expression of phospho-ERK1/2 has been normalized to expression in calnexin and quantified by densitometry. Lipin-1 translocation was detected with an anti-HA antibody (red fluorophore) and an antibody against the C-terminus of lipin-1 (green fluorophore) and the superposition of these two signals is shown as yellow bands. Phospho-ERK1/2 was detected with a green fluorophore-tagged antibody and ERK1/2 was detected with a red-fluorophore-tagged antibody and the superposition of the two signals is shown.

3.10. Preliminary evidence that lipin-2 does not translocate to membranes in response to treatment with lysophosphatidic acid or endothelin-1

We used digitonin lysis to separate MDA MB 231 cells into membrane and cytosolic fractions. We found no evidence of translocation of either endogenous (Fig. 3.9A) or overexpressed lipin-2 (Fig. 3.9B) to the membrane fraction. These two sets of western blotting were done in the same cells, one with ranges and the other with single values and both western blot analyses showed consistently there was no evidence of translocation of lipin-2 to membranes in response to stimulation with the PLD agonists LPA and endothelin-1 (Fig 3.9A-B). Furthermore, we observed that overexpressed lipin-2 is already on the membrane in MDA MB 231 cells. These results would argue against a major role for lipin-2 in regulating PLD signalling at the membrane but these results must be confirmed with larger sample sizes and statistical analysis.


A)
Inhibitor
Control Vehicle
50 nM ET-1
Pan PLD
PLD1 +ET1
PLD2 +ET1
DG Kinase +ET1
150
100
Lipin-2
100
Calnexin
Average Intensity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control vehicle
ET-1
Pan PLD inhibitor
PLD1 inhibitor
PLD2 inhibitor
DAGK inhibitor
Endothelin-1

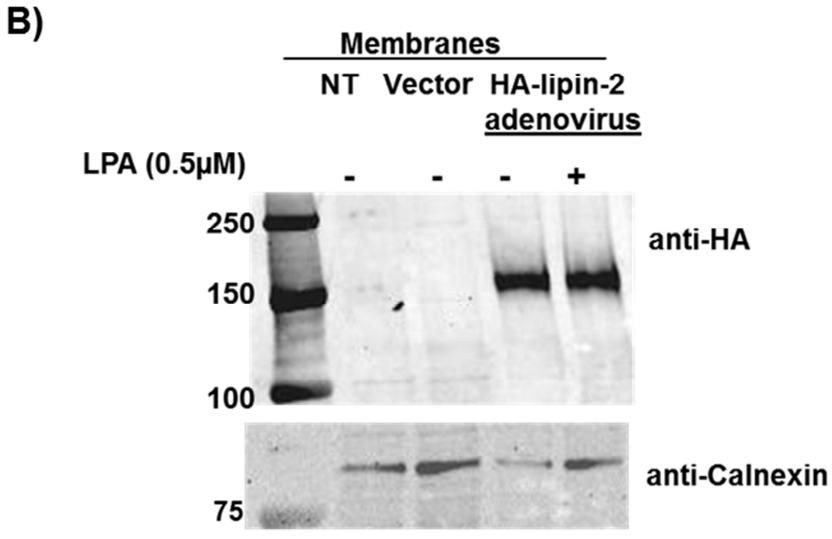

B)
Membranes
NT
Vector
HA-lipin-2 adenovirus
LPA (0.5μM)
- - - +
250
150
100
anti-HA
75
anti-Calnexin

Figure 3.9. Evidence that lipin-2 does not translocate to membranes in MDA MB 231 cells. A. Cells were pre-incubated with the indicated inhibitors for 30 min. Cells were then treated with endothelin-1 (ET-1) or vehicle for 5 min. followed by digitonin lysis. Mmebrane fractions were analyzed by western blot analysis. Data represent means ± ranges. Expression of endogenous lipin-2 was detected with an anti-lipin-2 antibody and normalized to expression in calnexin and analyzed by densitometry. **B. Cells were either not infected (designated as 'NT') or infected with HA-tagged lipin-2 adenovirus or with vector control adenovirus.** Cells were treated with 0.5 μM LPA for 2 min. followed by digitonin lysis and membrane fractions were collected and analyzed by western blotting. Translocation of HA-tagged lipin-2 to membranes was detected with an anti-HA antibody. Results are from one experiment.

3.11. Phosphatidic acid accumulation in breast cancer cells is due to phospholipase D1 and phospholipase D2 activity and to activity of Ca^{2+}-dependent diacylglycerol kinases

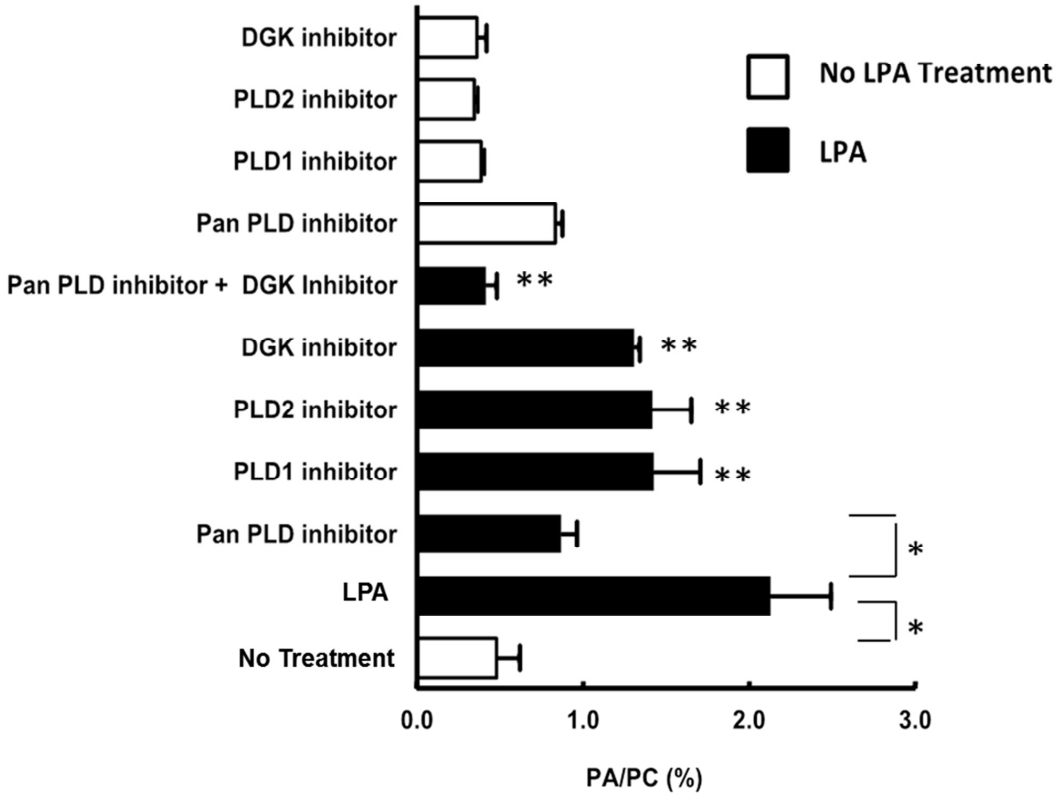
Although the MEFs are useful as a genetic model, we were unable to find an effect of either lipin-1 depletion or lipin-1 overexpression on ERK1/2 activation (Fig. 3.8). This result is consistent with previous studies in *fld* hearts [210] but contrary to other reports indicating that phosphorylation of ERK1/2 was upregulated in Schwann cells and in adipose tissue lacking lipin-1 expression [6,190]. Our result led us to hypothesize that the effect of lipin-1 on PA-dependent ERK1/2 phoshorylation was not easily detectable in MEFs and for this reason we attempted to characterize a role for the lipins in PLD-dependent signalling in cancer cell lines. We chose breast cancer cell lines because of reports linking increased DAG levels to increased PKC activation which was

associated with development of chemoresistance in breast cancer cell lines [249–251].

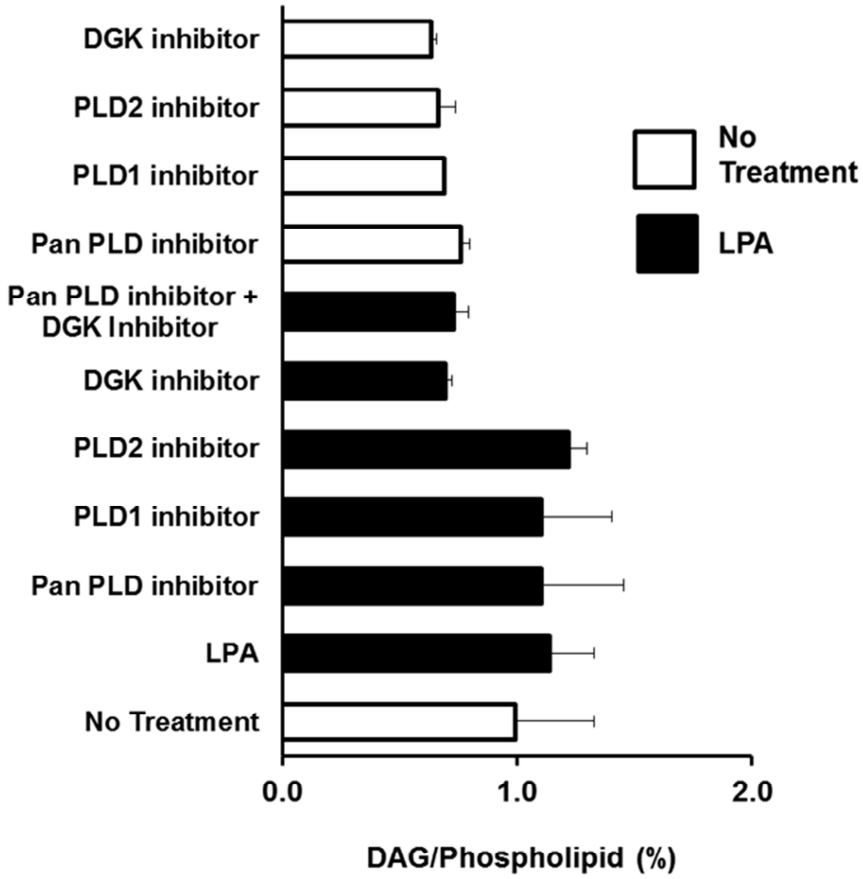
We found that most of the increase in PA can be attributed to PLD and DGK activity combined (Fig. 3.10A). The significant contribution of DGK to PA accumulation at 2 min. of stimulation with LPA could explain why DAG accumulation is not observed in these cells (Fig. 3.10B). LPA treatment increases labelling in PA in MCF-7 cells (Fig. 3.10A). In the breast cancer cell line MCF-7, treatment with chemical inhibitors of PLD1 and PLD2 caused a decrease in LPA-induced PA accumulation as measured by radiolabelling with [^{3}H]-palmitate (Fig. 3.10A). Cellular levels of DAG were not affected by LPA stimulation or inhibitors (Fig. 3.10B). However, PA can also be produced by the phosphorylation of DAG by DGKs [3]. Treatment of cells with a chemical inhibitor of Ca^{2+}-dependent DGK isoforms, R59949, prior to stimulation with LPA resulted in a decrease in PA accumulation (Fig. 3.10A). Treatment with both R59949 and the pan PLD isoform inhibitor resulted in a complete attenuation of the stimulation of PA accumulation by LPA stimulation (Fig. 3.10A). LPA treatment induced a 4-5 fold increase in the labelling incorporated into PA (Fig. 3.10A). PLD expression was confirmed by western blot analysis which detected endogenous PLD1 and PLD2 expression in MCF-7 cells infected with a vector control adenovirus (Fig. 3.10D). Infection with PLD1 or PLD2 adenovirus as a positive control confirmed detection of expression of both PLD isoforms (Fig. 3.10D).

We determined the optimal concentration of PLD1 and PLD2-specific inhibitors required to block PLD activity by preparing dose response curves showing the effect on inhibitor concentration on the PLD activity (Fig. 3.10E). We also measured bulk PA using the PA mass assay (Fig. 3.10C). Interestingly, when we measured the bulk cellular PA in one experiment, we found that LPA treatment promoted a 20-fold increase in bulk PA levels (Fig. 3.10C). Treatment with PLD1 or PLD2-specific inhibitors caused only a 15% decrease in the bulk concentration of PA in one experiment (Fig. 3.10C). Treatment with a PLD1/2 inhibitor caused only a 20% decrease in the bulk PA concentration in one experiment (Fig. 3.10C). These results provide preliminary evidence that bulk PA concentrations may be largely dependent on the activity of other signalling enzymes including DGK isoforms and $cPLA_2$. However, the experiment must be repeated so that the statistical significance of this effect can be determined.

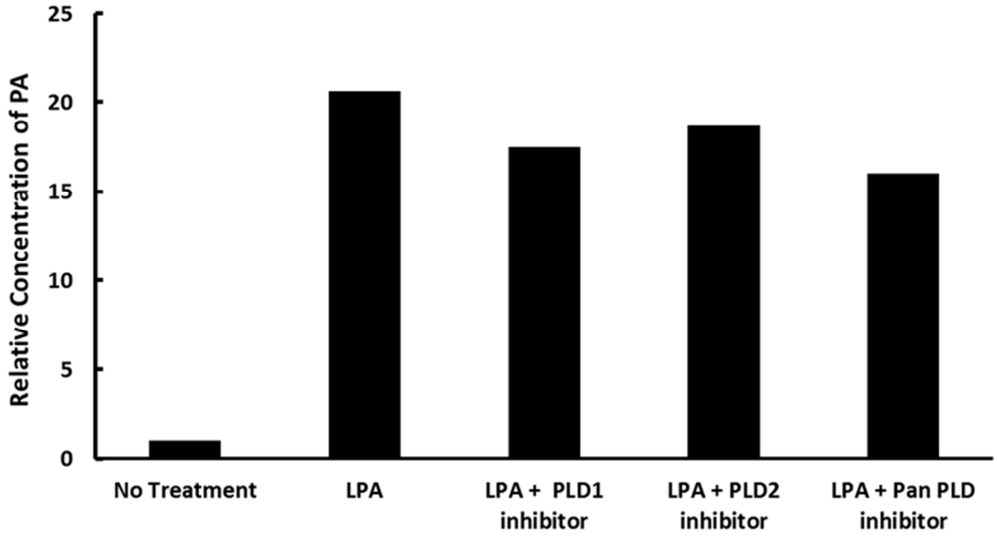
A)


DGK inhibitor
PLD2 inhibitor
PLD1 inhibitor
Pan PLD inhibitor
Pan PLD inhibitor + DGK Inhibitor
DGK inhibitor
PLD2 inhibitor
PLD1 inhibitor
Pan PLD inhibitor
LPA
No Treatment
No LPA Treatment
LPA
**
**
**
**
*
*
0.0
1.0
2.0
3.0
PA/PC (%)

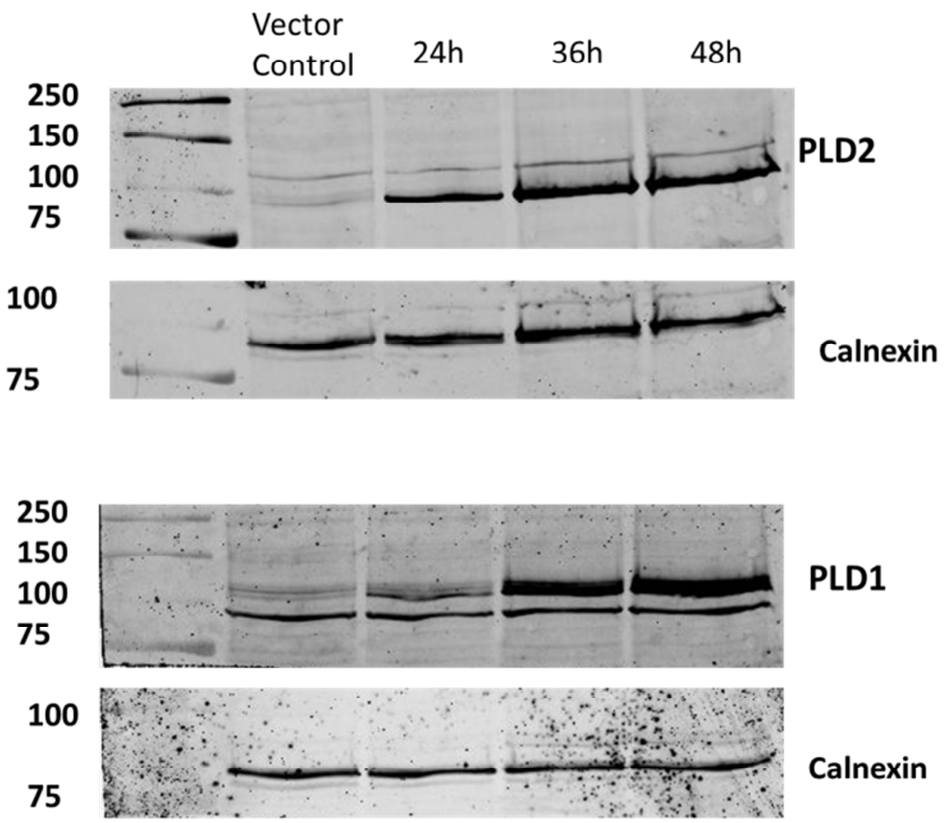
B)


DGK inhibitor
PLD2 inhibitor
PLD1 inhibitor
Pan PLD inhibitor
Pan PLD inhibitor + DGK Inhibitor
DGK inhibitor
PLD2 inhibitor
PLD1 inhibitor
Pan PLD inhibitor
LPA
No Treatment
No Treatment
LPA
0.0
1.0
2.0
DAG/Phospholipid (%)

C)


Relative Concentration of PA
25
20
15
10
5
0
No Treatment
LPA
LPA + PLD1 inhibitor
LPA + PLD2 inhibitor
LPA + Pan PLD inhibitor

D)


Vector Control
24h
36h
48h
250
150
100
75
PLD2
100
75
Calnexin
250
150
100
75
PLD1
100
75
Calnexin

E)

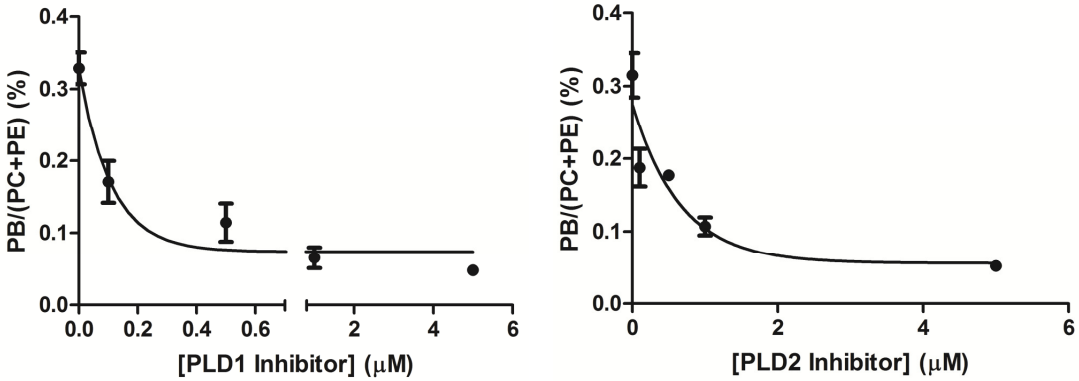

PB/(PC+PE) (%)
0.4
0.3
0.2
0.1
0.0
0.0
0.2
0.4
0.6
2
4
6
[PLD1 Inhibitor] (μM)
PB/(PC+PE) (%)
0.4
0.3
0.2
0.1
0.0
0
2
4
6
[PLD2 Inhibitor] (μM)

Figure 3.10. PLD activity is a major source for the PA accumulation in breast-cancer cells. A. PA accumulation in MCF-7 breast cancer cells is dependent upon PLD activity and activity of Ca^{2+}-dependent DAG kinases. Cells were pre-treated with inhibitor or vehicle for 30 min. followed by treatment with LPA or vehicle for 2 min. Significance is indicated as follows from Student's *t*-test: *, $p<0.001$; **, sample different from sample treated with LPA only ($p<0.005$). A one-way ANOVA gave a mean square between treatments of 0.000179 with $p<0.0001$. **B. DAG levels in MCF-7 cells. Cells were pre-treated with inhibitor or vehicle for 30 min. followed by treatment with LPA or vehicle for 2 min.** Means of samples treated with inhibitors and LPA are not significantly different from sample treated with LPA only or from untreated control. A and B, values represent means ± S.E.M. from 6 independent observations per treatment. Variance was determined from a one-way ANOVA. For A and B, Pan PLD inhibitor was used at a concentration of 1 µM and PLD1 or PLD2 inhibitors were used at a concentration of 0.5 µM. **C. PA accumulation in MDA MB 231 cells is PLD-dependent as measured by the PA mass assay.** MDA MB 231 cells were treated with 10 µM LPA for 2 min and pretreated with inhibitor or vehicle for 30 min. PLD1 or PLD2 inhibitor was used at a concentration of 0.5 µM. Pan PLD inhibitor was used at a concentration of 1 µM. The bulk concentration of PA was determined relative to the intensity of the no treatment control which was set to a value of 1. Intensity of staining in the bands was determined from the TLC plate by densitometry using Odyssey Infrared Imaging software. Results are from one experiment. The white columns received no treatment except where indicated and the black columns received LPA treatment. The inhibitor-only negative controls were compared to the no treatment control. There were no statistical differences found between any of the inhibitor-only controls and the no treatment control. **D. Western blot of PLD1 and PLD2 endogenous levels in cells infected with a vector control and adenoviral overexpression of PLD1 and PLD2 as a positive control in MCF-7 cells from one experiment. E. Concentration curves for the effect of PLD1 and PLD2 inhibitors on endogenous PLD activity in MEFs.** Cells were not treated with agonist. Values represent means ± ranges**.** Curve fitting analysis was performed using GraphPad prism.

3.12. Doxorubicin-resistant MCF-7 cells show less phosphatidic acid accumulation than syngenic control cells

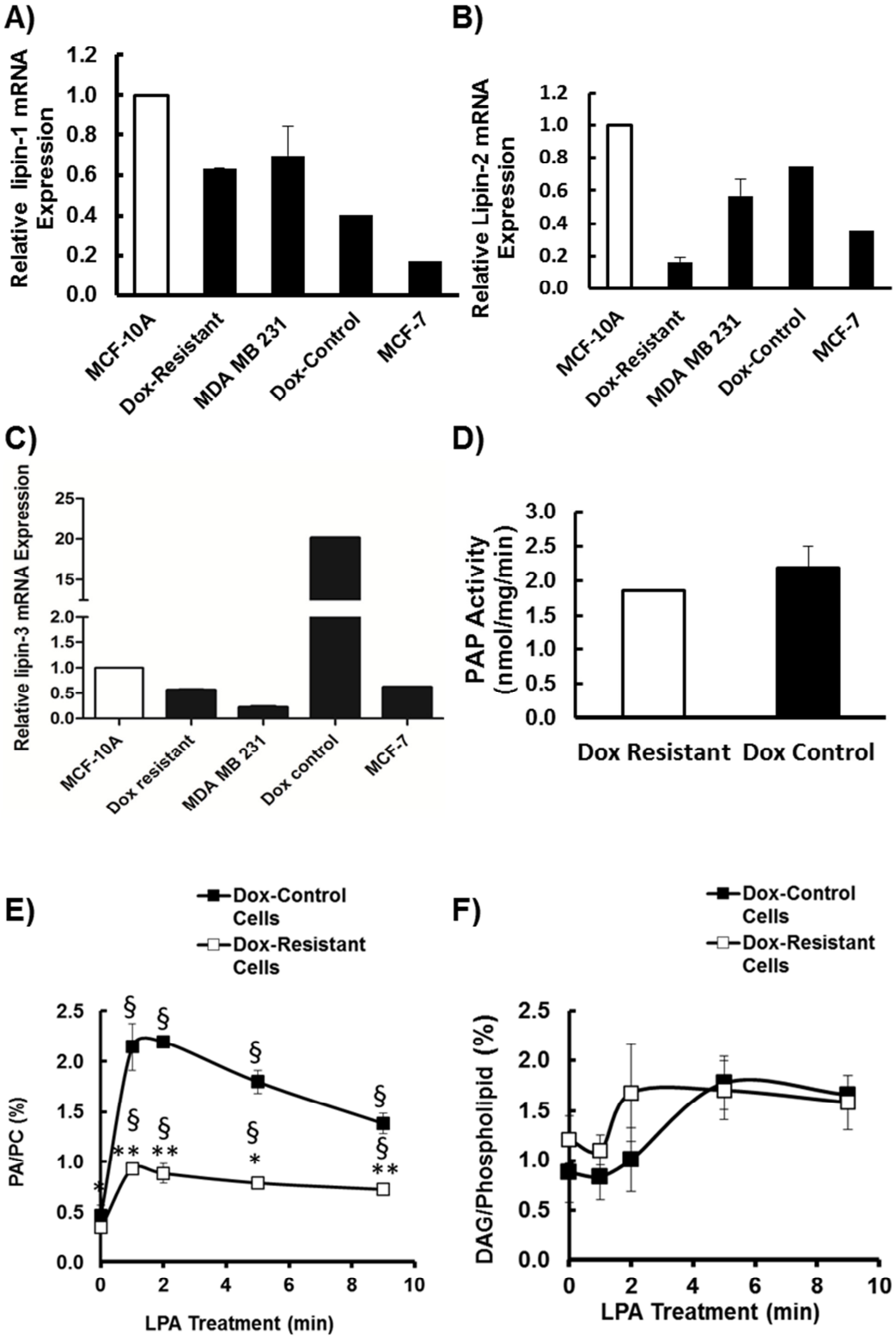
We hypothesized that protection against the cytotoxic effects of Doxorubicin (Dox) could be conferred by LPA-induced activation of NF-κB through PLD activation. Dox is a commonly used anti-cancer drug that is an anthracycline antibiotic [252]. However, Dox-resistance often develops rapidly in cancer patients and limits the efficacy of this drug [244]. Given that we have found evidence that more PAP activity is associated with membranes in response to PLD activation in a variety of cancer cell lines (Fig. 3.7), we asked whether PAP activity could promote or inhibit chemoresistance in cancer cells. The chemotherapeutic agent Dox promotes apoptosis at the dose used in selection of resistance [253]. Our goal was to determine whether PA accumulation which promotes cell survival was a factor in Dox resistance in breast cancer cells [254].

We observed differential expression of the lipins in Dox-resistant cells compared to control cells (Fig. 3.11A-C). mRNA expression of all three lipin isoforms was also detected and was normalized to the lipin expression of other cell lines (Fig. 3.11 A-C). Results from one to two experiments showed that lipin-1 expression is decreased in both Dox-resistant and Dox-control MCF-7 cells and in other cell lines including MDA MB 231 as compared to the normal breast epithelial cell line MCF-10A (Fig. 3.11A). The same trend of decreased expression in the cancer cell lines was observed for lipin-2 (Fig. 3.11B), whereas Dox-control cells showed very high expression of lipin-3 (Fig. 3.11C).

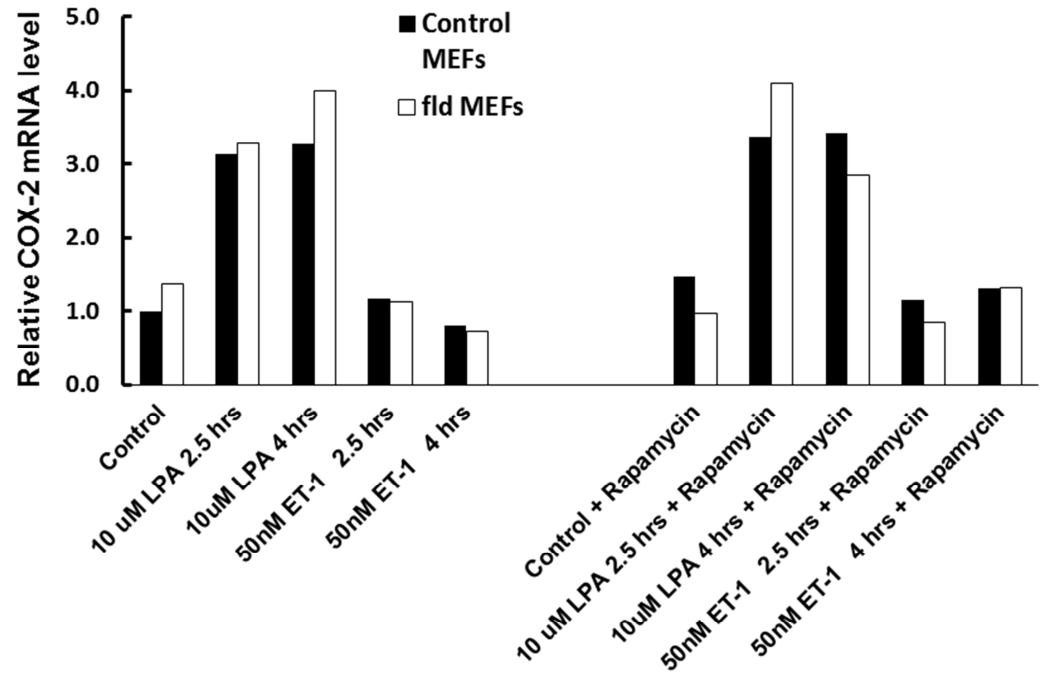
To further examine the importance of this result, we pre-labelled control MCF-7 and Dox-resistant MCF-7 cells with [^{3}H]-palmitate and measured labelling in PA and DAG (Fig. 3.11D-E). In these experiments, cells were pre-labelled with [^{3}H]-palmitate for 2 h. We then treated Dox-resistant and Dox-control MCF-7cells with LPA for the indicated time points (Fig. 3.11D-E). PA accumulation was time-dependent and DAG accumulation occurred after PA accumulation had begun to decline in a time-dependent manner (Fig. 3.11D-E). The spike in PA levels occurred at 1 min and preceded the peak in DAG levels which occurred at 2 min in Dox-resistant cells and at 5 min in Dox-control cells (Fig. 3.11D-E). The conversion of PA to DAG by PAP was more rapid in Dox-resistant cells than in controls (Fig. 3.11D-E). In Dox-control cells, PA accumulation peaked at 1.5 min of LPA treatment whereas DAG accumulation peaked at 5 min (Fig. 3.11D). In contrast, DAG accumulation peaked at 2 min in Dox-resistant cells and follows a plateau from 2 min to 5 min (Fig. 3.11E). We did not find upregulation of PA levels in Dox-resistant cells as compared to controls (Fig. 3.11D). In contrast, we observed that PA levels were increased in Dox control cells (Fig. 3.11D). DAG levels were only significantly decreased (p<0.05) in Dox-resistant cells at 2 min of LPA treatment and for all other time points there were no significant differences between Dox-resistant and Dox control cells in DAG labelling, although there was a trend for increased DAG levels in Dox-resistant cells at time points preceding 2 min of treatment (Fig. 3.11E). We found a significant increase in PA accumulation in controls and consistently we found a trend for increased DAG levels in the resistant cells (Fig. 3.11D-E). However, PAP activity in Dox-resistant as compared to control cells was not altered (Fig. 3.11F). We

concluded from these experiments that increased DAG levels were associated with MCF-7 resistance cells whereas PA accumulation did not appear to be associated with signalling promoting the development of Dox-resistance.

We therefore questioned how DAG levels could promote signalling leading to resistance and hypothesized that the effect could be through PKC signalling effects on transcriptional upregulation of COX-2 in macrophages or of NF-κB in breast cancer cell lines. We performed one RT-PCR experiment in *fld* and control MEFs in which we measured COX-2 and NF-κB mRNA expression (Fig. 3.11G-H). Although there was no effect on COX-2 mRNA expression in *fld* as compared to control MEFs, we found that NF-κB mRNA expression was consistently downregulated under basal conditions and regardless of LPA or with rapamycin treatment to determine whether mTORC1 could be regulating the signaling (Fig. 3.11G-H). This suggested there may be a possible effect of PAP activity of lipin-1 in promoting increased DAG levels and increased DAG signalling which could promote a signalling cascade leading to upregulation of NF-κB through activation of PKC by DAG. Alternatively, lipin-1 could act in the nucleus as a transcription factor to promote transcription of the NF-κB gene. To substantiate one of these interpretations will require further testing. However, we did not find an effect of rapamycin on the mRNA expression of NF-κB or COX2 (Fig. 3.11G-H).


A)
Relative lipin-1 mRNA Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MCF-10A
Dox-Resistant
MDA MB 231
Dox-Control
MCF-7
B)
Relative Lipin-2 mRNA Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MCF-10A
Dox-Resistant
MDA MB 231
Dox-Control
MCF-7
C)
Relative lipin-3 mRNA Expression
25
20
15
2.0
1.5
1.0
0.5
0.0
MCF-10A
Dox resistant
MDA MB 231
Dox control
MCF-7
D)
PAP Activity (nmol/mg/min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Dox Resistant
Dox Control
E)
Dox-Control Cells
Dox-Resistant Cells
PA/PC (%)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
LPA Treatment (min)
F)
Dox-Control Cells
Dox-Resistant Cells
DAG/Phospholipid (%)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
LPA Treatment (min)

G)


Relative COX-2 mRNA level
5.0
4.0
3.0
2.0
1.0
0.0
Control
MEFs
fld MEFs
Control
10 uM LPA 2.5 hrs
10uM LPA 4 hrs
50nM ET-1 2.5 hrs
50nM ET-1 4 hrs
Control + Rapamycin
10 uM LPA 2.5 hrs + Rapamycin
10uM LPA 4 hrs + Rapamycin
50nM ET-1 2.5 hrs + Rapamycin
50nM ET-1 4 hrs + Rapamycin

H)

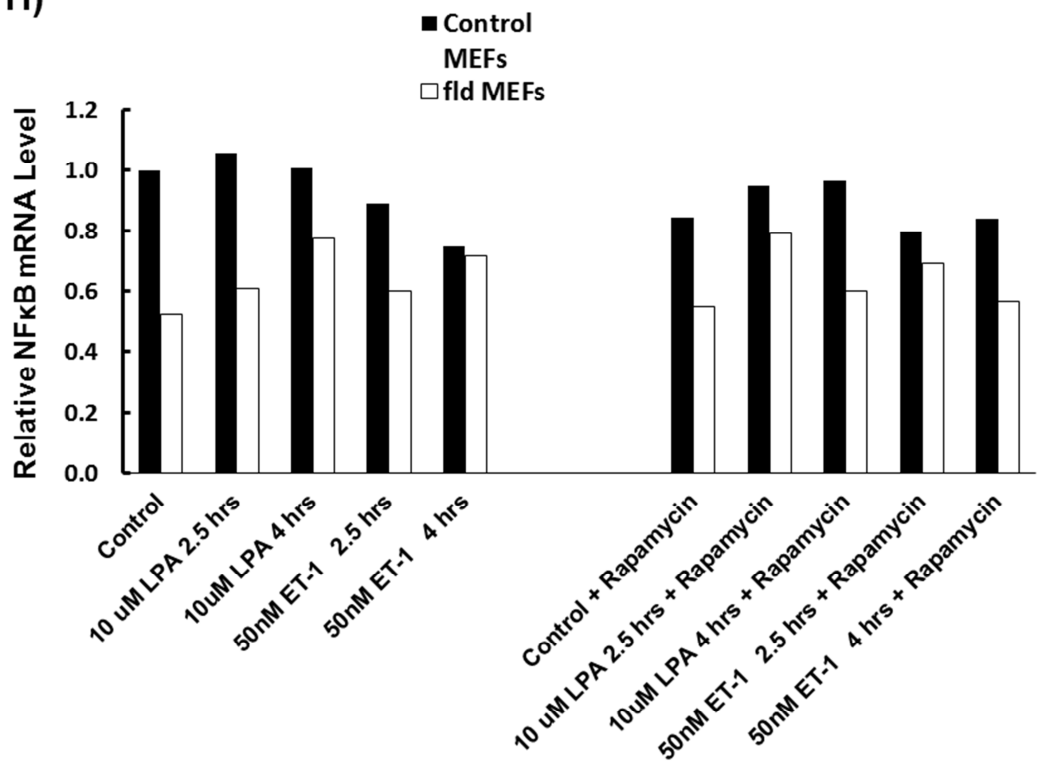

Relative NFκB mRNA Level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
MEFs
fld MEFs
Control
10 uM LPA 2.5 hrs
10uM LPA 4 hrs
50nM ET-1 2.5 hrs
50nM ET-1 4 hrs
Control + Rapamycin
10 uM LPA 2.5 hrs + Rapamycin
10uM LPA 4 hrs + Rapamycin
50nM ET-1 2.5 hrs + Rapamycin
50nM ET-1 4 hrs + Rapamycin

Figure 3.11. PA accumulation is greater in control MCF-7 cells than in Dox-resistant MCF-7 cells. **A-C. mRNA expression of the 3 lipin isoforms as measured by RT-PCR in breast cancer cell lines and normal breast epithelial cells.** The lipin mRNA expression levels were normalized to mRNA levels of cyclophilin A and the MCF-10A expression is set to a value of 1. Relative mRNA levels of each cell line are shown in comparison to the mRNA level of MCF-10A cells. **D. PAP activity in Dox-resistant and Dox control cells.** Error bars represent means ± ranges from two independent observations per treatment and the other values are from one experiment. **E. PA labelling in Dox-resistant and Dox control cells.** A Repeated Measures analysis of variance with time as the repeated measure gave $p<0.0001$ and a mean square of 0.171 for Dox-resistant cells and $p<0.0001$ and a mean square of 1.538 for Dox-control cells. Significance is indicated as follows: *, Dox-resistant different from control ($p<0.05$), **, Dox-resistant different from control ($p< 0.001$), §, different from untreated control ($p<0.001$). **F. DAG labelling in Dox-resistant and Dox control cells.** The means were not significantly different. For A and B, values represent means ± S.D. from 3 to 6 independent observations per treatment. **G. mRNA expression of COX-2 as measured by RT-PCR in fld and control MEFs.** Cells were treated with or without 50 nM of rapamycin. The COX-2 mRNA expression levels were normalized to mRNA levels of cyclophilin A and are from one experiment. Values from the control MEFs that received no treatment were set at a value of 1. **H. mRNA expression of NF-κB as measured by RT-PCR in *fld* and control MEFs.** The NF-κB expression levels were normalized to mRNA levels of cyclophilin A and are from one experiment and are set to a value of 1 in the control MEFs that received no treatment. Cells were treated with 50 nM of rapamycin or with vehicle.

3.13. The chemical inhibitor of lipins, BEL, promotes phospholipase D-mediated cell migration but does not affect phosphatidic acid accumulation

Since we determined that increased PAP activity is associated with membranes in response to PLD activation, we investigated the relevance of this association in relation to cell signalling and cell migration. We used Rat2 fibroblasts as a model for cell migration because previous work showed that Rat2 fibroblasts migrate in response to LPA and endothelin-1, the PLD agonists which promoted the increased association of PAP activity at the membrane [131].

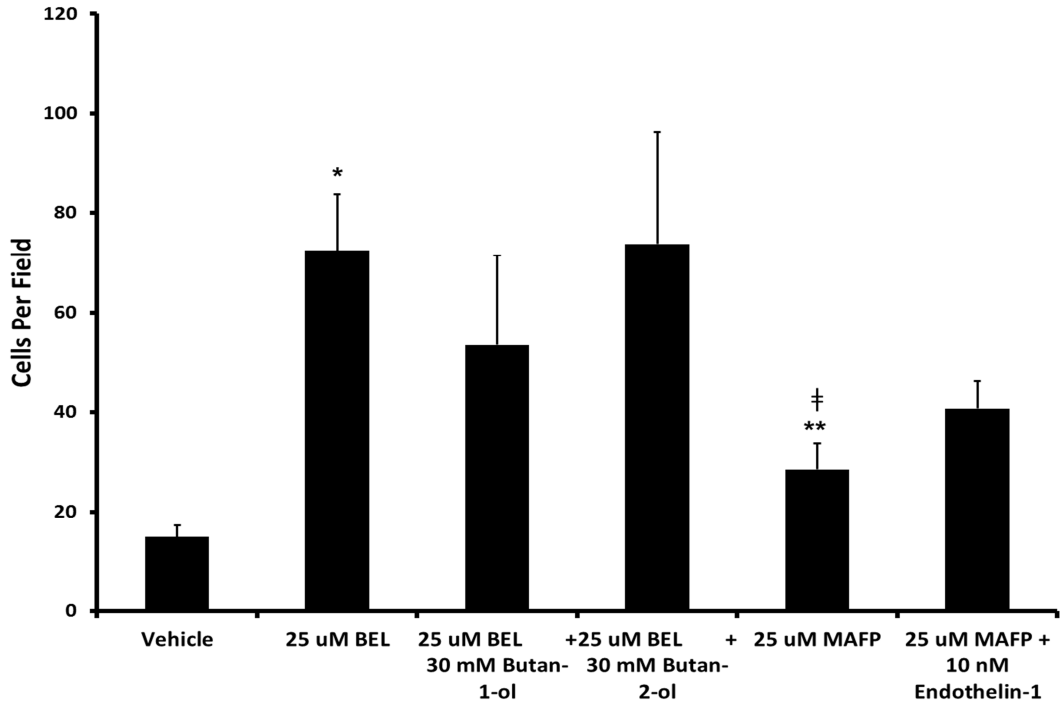
We performed transwell assays to determine whether the cell migration induced by endothelin-1 is PLD-dependent using the primary alcohol butan-1-ol to inhibit PA formation by PLD. Butan-1-ol acts as a nucleophilic acceptor in the transphosphatidylation reaction catalyzed by PLD whereas butan-2-ol is not a substrate for the transphosphatidylation reaction catalyzed by PLD and should not inhibit PA formation [54]. Using the butan-2-ol treatment as the negative control, we showed that the effects of endothelin-1 in promoting migration of Rat2 fibroblasts were partially attenuated by treatment with butan-1-ol but not with butan-2-ol indicating that endothelin-1 stimulates migration in a PLD-dependent manner (Fig. 3.12A). As a control, we showed that treatment with butan-1-ol or butan-2-ol alone did not affect cell migration (Fig. 3.12B). To examine whether the PAP activity of lipins could attenuate or promote PLD-mediated migration, we further tested to see whether BEL, the chemical inhibitor of lipin, could attenuate or promote PLD-mediated migration (Fig. 3.12B). BEL is an inhibitor of both PAP activity and $cPLA_2$ activity, whereas the chemical inhibitor methyl arachidonyl

fluorophosphonate (MAFP) inhibits mostly $cPLA_2$ activity but not PAP activity and is a control for $cPLA_2$-inhibitory effects of BEL [255]. We found that BEL promoted a significant increase in cell migration ($p<0.01$) as compared to the inhibitor MAFP, which does not inhibit lipin activity (Fig. 3.12B). These transwell migration experiments (Fig. 3.12A-B) must be repeated to test whether there is a significant inhibitory effect of BEL on endothelin-1-stimulated migration. However, neither treatment with BEL alone nor treatment with MAFP alone significantly affected the accumulation of PA (Fig. 3.12C). Moreover, labelling in PA measured as PA/PC (%) was consistent with labelling in PA measured as PA/total phospholipid (%), which further supported our measurements of PLD-dependent PA accumulation (Fig. 3.12C). Moreover, there was no significant difference in PA accumulation between endothelin-1 treated cells and cells treated with endothelin-1 and BEL (Fig. 3.12C). We also used E600, a chemical inhibitor of lipases, to inhibit the degradation of DAG by lipases (Fig. 3.12D). We found that treatment with E600 in combination with a DGK inhibitor did not affect DAG accumulation (Fig. 3.12D).

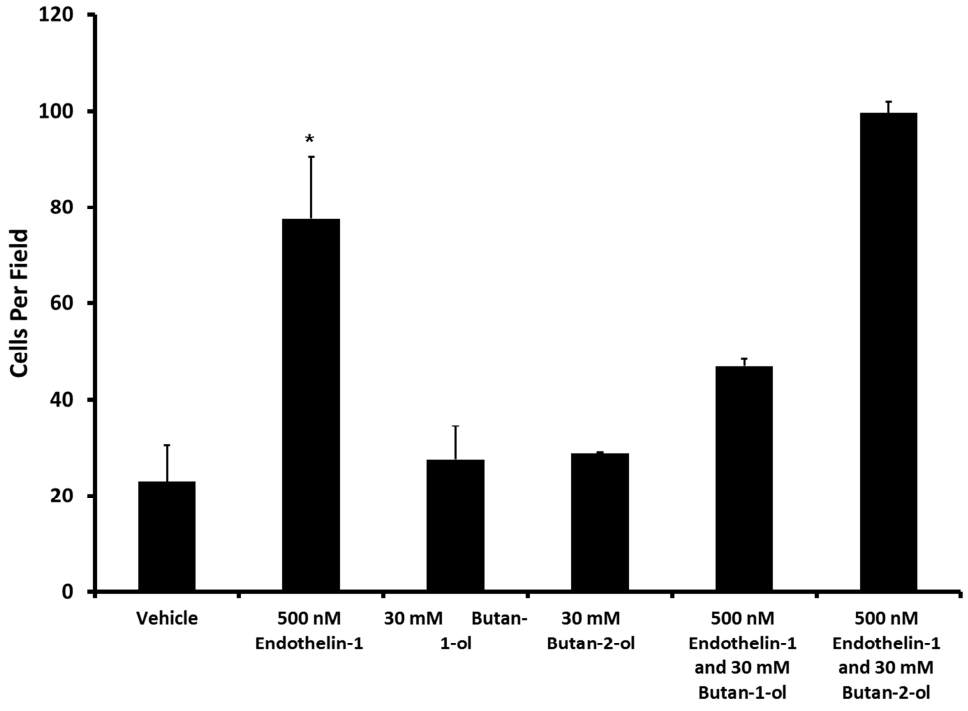
We were unable to test the effect of lipin-1 depletion more directly on PLD-dependent cell migration in the MEF model because neither the *fld* nor the control MEFs were found to migrate in response to endothelin-1 as compared to Rat2 fibroblasts (Fig. 3.12E). We used western blot analysis to show that treatment with BEL did cause increased phosphorylation of ERK1/2, but this effect occurred in the absence of LPA and treatment with MAFP caused a similar effect (Fig. 3.12F). We also treated cells with PD98059, a chemical inhibitor of

ERK1/2 phosphorylation, which caused a decrease in phosphorylation (Fig. 3.12F). Moreover, treatment of cells with BEL and butan-1-ol or BEL and butan-2-ol caused in both cases a small abrogation of ERK1/2 activation so that we cannot demonstrate that the effect of BEL in promoting cell migration and ERK1/2 phosphorylation is dependent upon PLD signalling. This western blot analysis will need to be repeated so that statistical analysis can be determined. Overall, these results suggested that enzymatic activity other than that of PAP activity such as that of $cPLA_2$ activity, could be responsible for promoting cell migration by increasing PA accumulation on the membrane. This result is consistent with our finding that treatment with BEL did not affect endothelin-1 induced PA accumulation (Fig. 3.12C-D). We also verified that lipin-1 and lipin-3 isoforms are expressed in fibroblasts (Fig. 3.12G). We concluded that PA accumulation appears to be important in promoting migration in Rat2 fibroblasts. However, we could not refute an effect of lipins on cell migration because the mechanism of BEL might not be specific for lipins and the MEFs did not show migration in response to PLD agonists.

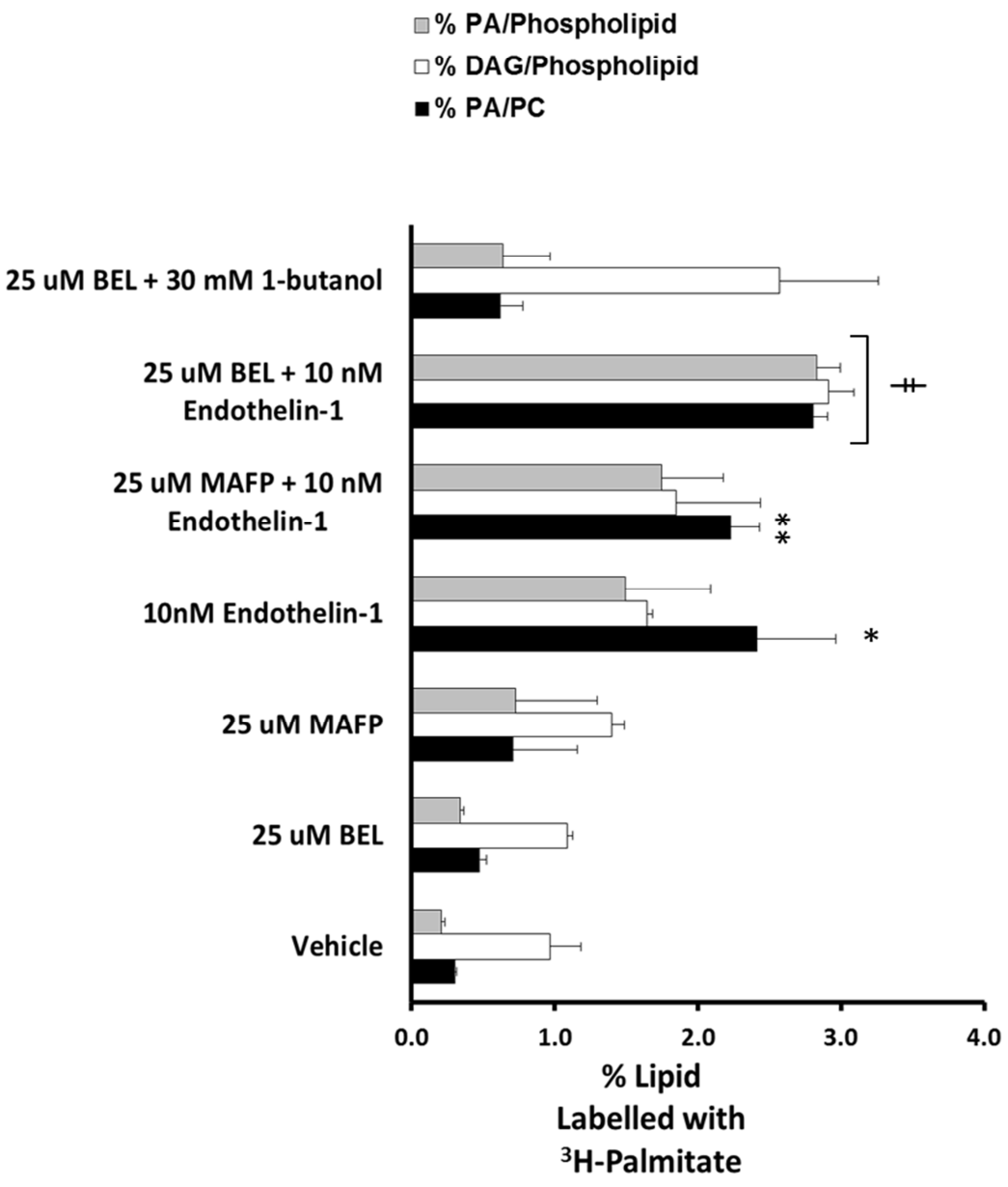
A)


120
100
80
60
40
20
0
Cells Per Field
*
‡
**
Vehicle
25 uM BEL
25 uM BEL 30 mM Butan-1-ol
+25 uM BEL 30 mM Butan-2-ol
+ 25 uM MAFP
25 uM MAFP + 10 nM Endothelin-1

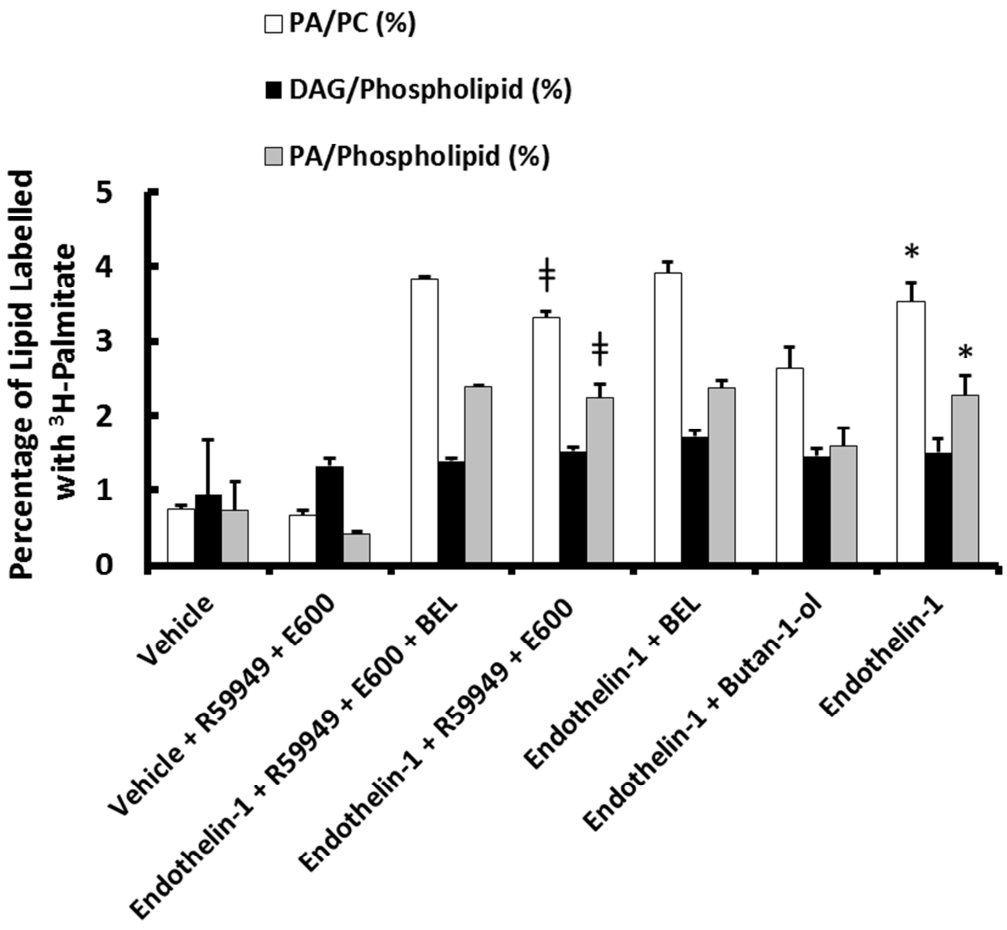
B)


120
100
80
60
40
20
0
Cells Per Field
*
Vehicle
500 nM Endothelin-1
30 mM Butan-1-ol
30 mM Butan-2-ol
500 nM Endothelin-1 and 30 mM Butan-1-ol
500 nM Endothelin-1 and 30 mM Butan-2-ol

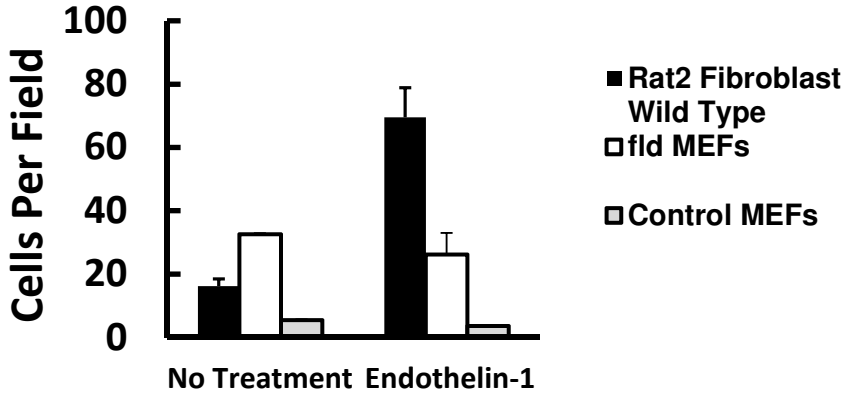
C)


% PA/Phospholipid
% DAG/Phospholipid
% PA/PC
25 uM BEL + 30 mM 1-butanol
25 uM BEL + 10 nM Endothelin-1
25 uM MAFP + 10 nM Endothelin-1
10nM Endothelin-1
25 uM MAFP
25 uM BEL
Vehicle
0.0
1.0
2.0
3.0
4.0
% Lipid Labelled with ^{3}H-Palmitate

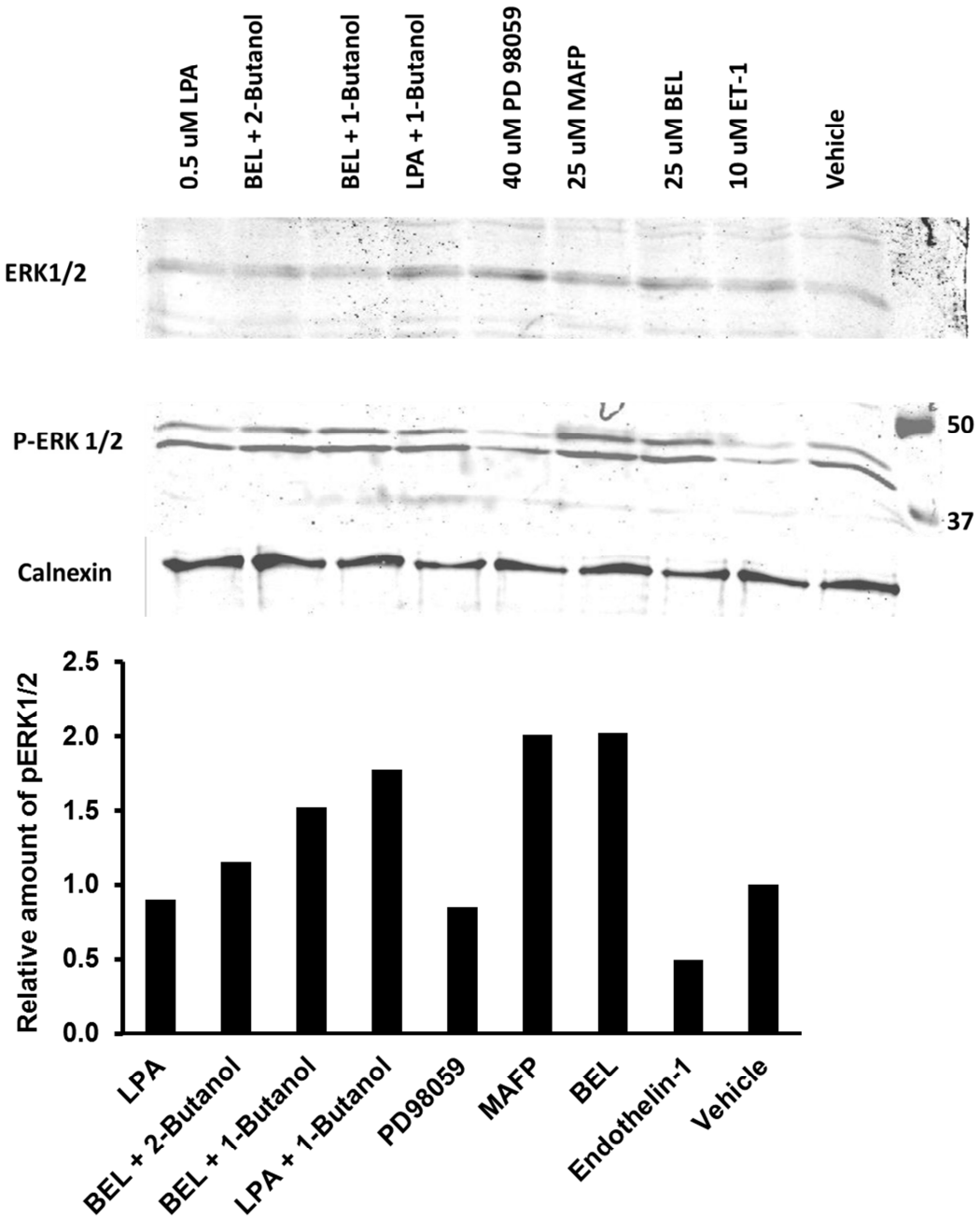
D)


PA/PC (%)
DAG/Phospholipid (%)
PA/Phospholipid (%)
Percentage of Lipid Labelled with ^{3}H-Palmitate
0
1
2
3
4
5
‡
‡
*
*
Vehicle
Vehicle + R59949 + E600
Endothelin-1 + R59949 + E600 + BEL
Endothelin-1 + R59949 + E600
Endothelin-1 + BEL
Endothelin-1 + Butan-1-ol
Endothelin-1

E)


Cells Per Field
0
20
40
60
80
100
No Treatment
Endothelin-1
Rat2 Fibroblast Wild Type
fld MEFs
Control MEFs

F)


0.5 uM LPA
BEL + 2-Butanol
BEL + 1-Butanol
LPA + 1-Butanol
40 uM PD 98059
25 uM MAFP
25 uM BEL
10 uM ET-1
Vehicle
ERK1/2
P-ERK 1/2
50
37
Calnexin
Relative amount of pERK1/2
2.5
2.0
1.5
1.0
0.5
0.0
LPA
BEL + 2-Butanol
BEL + 1-Butanol
LPA + 1-Butanol
PD98059
MAFP
BEL
Endothelin-1
Vehicle

G)

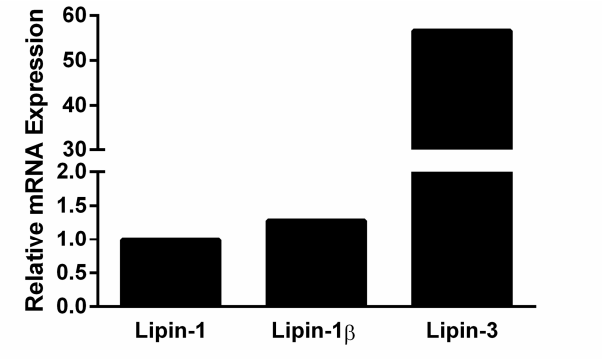


Figure 3.12. The BEL inhibitor promotes PLD-dependent cell migration in the transwell assay but does not affect PA accumulation. Experiments were done with Rat2 fibroblasts except where indicated. A. Cell migration in Rat2 fibroblasts is activated by endothelin-1 and is also PLD-dependent. B. Cell migration is activated by treatment with BEL alone but not with the control inhibitor MAFP. For A and B, values represent means ± ranges or means ± SEM from 4 independent observations per treatment and statistical analysis was performed on the means from 4 independent observations. Statistical significance is indicated as follows: *, sample is significantly different from vehicle-treated control (p<0.01), ǂ, sample is significantly different from sample treated with BEL only (p<0.01), **, sample is significantly different from MAFP and endothelin-1-treated sample (p<0.05). **C. BEL does not increase PA accumulation in the presence or absence of treatment with endothelin-1.** *, sample is significantly different from vehicle-treated control (p<0.01), **, significantly different from MAFP-treated control (p<0.01), ǂ, significantly different from sample treated with endothelin-1 only (p<0.05). **D. PA and DAG labelling is not affected by treatment with chemical inhibitors of DGK or by E600, a lipase inhibitor.** *, significantly different from vehicle-treated control (p<0.01), ǂ, significantly different from sample treated with vehicle and R59949 and E600 (p<0.01). For figures C-D, values represent means ± S.E.M. from 4 independent observations per treatment. **E. Control and *fld* MEFs do not migrate towards endothelin-1 as compared to Rat2 fibroblasts.** Values represent means ± ranges. **F. BEL upregulates the activation of ERK1/2 as determined from western blot analysis from one experiment.** Cells were treated with PLD agonists LPA or endothelin-1 at the indicated concentrations. Total cell lysates were analyzed by western blotting. Bands were quantified by densitometry and normalized to expression of calnexin. **G. mRNA expression of lipin isoforms as measured by RT-PCR in Rat2 fibroblasts.** The lipin mRNA expression levels were normalized to mRNA levels of cyclophilin A and the lipin-1 mRNA expression is set to a value of 1.

3.14. Discussion

Evidence discussed in the introduction showed that PAP activity could play a role in the PLD pathway by metabolizing the PA produced by PLD. The identities of the enzymes that metabolize PA produced by PLD signalling in pathology have not yet been conclusively determined. This work represents the first examination of the role of lipins in PLD-dependent signalling in breast cancer cells. We have also utilized *fld* MEFs as a genetic model to reveal the contribution of lipin-1 to signalling via PLD. Much previous work showed that PAP activity showed increased association with membranes in response to stimulation with LPS and was PLD-dependent [237,238,256]. In this work we provide evidence that increased PAP activity is associated with membranes in cancer cells. However, lipin-1 did not show PLD-activated translocation in *fld* MEFs and lipin-2 did not show translocation in breast cancer cells (Fig. 3.6-3.8). The relevance of the translocation of lipin-1 onto membranes in cancer cells may arise from increased DAG levels on membranes which could activate NF-κB, possibly through PKC [240,249,256]. We showed from one experiment that NF-κB expression is lower in *fld* compared to control MEFs but this finding needs to be verified by doing more experiments so that significance can be determined (Fig. 3.11H). On the other hand, we found that in MEFs and cancer cells, PA accumulation appeared to be PLD-dependent but was independent of lipin-1 or lipin-2 expression (Fig. 3.4-3.5). Specifically, depletion or overexpression of lipin-1 or overexpression of lipin-2 did not affect PA levels (Fig. 3.4-3.5). These results lead to the conclusion that the accumulation of the bulk signalling lipid PA

in MEFs was not regulated by lipin-1 expression and rather may be regulated by other enzymes including DGK isoforms and $cPLA_2$ enzymes. In order to investigate this question we attempted to study the regulation of PLD signalling by lipin-1 or lipin-2 using genetic approaches. In this Chapter, we determined that a lack of lipin-1 expression was not compensated for in MEFs by upregulation of lipin-2 or lipin-3 mRNA expression at a statistically significant level (Fig. 3.1A). Previous work in LPP1 knockout MEFs showed increased PLD activity compared to controls expressing LPP1 and this led some to question how LPPs could regulate PA derived from PLD [131]. However, we found no significant difference in bulk PC labelling between *fld* and control MEFs (Fig.3.3A) or in PLD activation from studies with means ± ranges from two experiments (Fig. 3.3B-C). We found that lipin-1 does not significantly affect regulation of PC metabolism within MEFs (Fig. 3.3A). If increased PLD activity were observed in *fld* compared to control MEFs, this could indicate that lipin-1 plays a direct role in negatively regulating PLD activity.

The catalytic activity of lipin-1 in MEFs is redundant and could be compensated for by other lipins, which show mRNA expression in MEFs (Fig. 3.1A-B). We found that lipin-2 or lipin-3 or both isoforms can substitute/replace the PAP activity of lipin-1 because the *fld* MEFs show no decrease in PAP activity compared to control MEFs (Fig. 3.1B). However, the mechanism for redundancy of PAP activity of lipins does not appear to involve reciprocal upregulation of lipin-2 or lipin-3 expression in *fld* MEFs as determined by measurements of mRNA levels of lipins in *fld* and control MEFs (Fig. 3.1A).

However, there was a trend for increased lipin-2 expression in *fld* MEFs as compared to controls (Fig. 3.1A). It is possible that a much larger sample size might reveal compensatory upregulation of lipin-2 or lipin-3 expression in *fld* MEFs. In contrast, increased relative mRNA levels of lipin-2 and lipin-3 isoforms were detected in lipin-1 knockout hearts as compared to controls [210]. Also in contrast to studies in HeLa cells which found upregulation of lipin-2 mRNA expression in lipin-1 depleted cells and similar upregulation of lipin-1 in lipin-2-depleted cells [173], depletion of each lipin isoform did not result in increased expression of the other isoforms (Fig. 3.1A). PAP activity in *fld* MEFs was also not significantly decreased compared to control MEFs and this provides evidence for redundancy of PAP activity in some cell types across the lipin isoforms (Fig. 3.1B). Previous studies in *fld* mice showed that kidney, liver, brain, and skeletal muscle from these mice have significantly less PAP activity than in wild type mice and previous studies in *fld* hearts found a significant decrease in PAP activity in hearts of *fld* mice compared to hearts of control mice [183,210]. Knockout of both lipin-1 and lipin-2 in mice also caused embryonic lethality [257].

We utilized a knockdown model to test for compensation of lipin expression. Knockdown with siRNA was done for lipin-1, lipin-2, and lipin-3 in the MDA MB 231 breast cancer cell line (Fig. 3.2A-C) in order to further investigate the mechanism of redundancy between lipin isoforms. The lack of upregulation of lipin-2 or lipin-3 mRNA expression that we found in *fld* MEFs (Fig. 3.1A) is supported by our experiments in which each lipin isoform was knocked down and there was no upregulation of mRNA expression of the other lipin isoforms (Fig.

3.2A-C). Redundancy of function of the lipins is supported by several previous studies, which showed that lipins are present in excess and there is PAP activity remaining when the expression of lipin isoforms is depleted [210,257]. A 40-50% efficiency of knockdown of each isoform individually was achieved and this represented a significant knockdown at $p<0.01$ (Fig. 3.2A-C). The next step for this work would be to determine which lipin isoform is the major PAP enzyme in these breast cancer cells by measuring the PAP activity in cells in which individual lipin isoforms have been knocked down and in cells with a triple knockdown of all three lipins.

We labelled bulk cellular PA and DAG (Fig. 3.4-3.5). The PA labelling experiments in *fld* and control (heterozygous) MEFs did not show statistically significant differences between *fld* and control MEFs (Fig. 3.4A) and are consistent with redundancy between the lipin isoforms we have observed (Fig. 3.1B). Lipin-2 and lipin-3 are expressed in the *fld* and control MEFs (Fig. 3.1A) and have PAP activity which can prevent the hypothesized increase in PA accumulation in *fld* MEFs by catalyzing the turnover of PA following PLD activation (Fig. 3.4A, 3.5A-B). At 5 nM of endothelin-1 treatment there is a significant increase in DAG labelling ($p<0.05$) in the controls as compared to the *fld* MEFs, which is consistent with our hypothesis of DAG being decreased in *fld* MEFs (Fig. 3.4B). However, none of the other concentrations of endothelin-1 show significant differences between *fld* and control MEFs in DAG labelling and the sample size of n = 9-15 is large enough for our conclusions to be reliable. This result is consistent with previous labelling studies of the PLD pathway in

Rat2 fibroblasts using stimulation with physiological PLD agonists such as LPA or EGF [131,225]. The unexpectedly high PAP activity in *fld* MEFs (Fig. 3.1B) could be from lipin-2 or lipin-3 isoforms. Dwyer *et al* demonstrated that lipin-1/lipin-2 double knockouts are embryonic lethal compared to lipin-1 or lipin-2 single knockout embryos which are viable, thus suggesting interchangeable roles of lipin-1 and lipin-2 during development of the mouse embryo [257].

We found that only with treatment at the two highest concentrations of endothelin-1 did overexpression of lipin-1 cause significantly increased DAG accumulation (Fig. 3.5B). An increase in DAG levels promoted by overexpression of lipin-1 was not consistently found at other concentrations of endothelin (Fig. 3.5B) which led to the conclusion that lipin-1 overexpression did not cause a consistent increase in DAG levels and this conclusion was consistent with previous evidence showing that the turnover of DAG is rapid in many cell types including MEFs [131]. This result is consistent with our finding that control MEFs did not consistently show significantly higher DAG levels than *fld* MEFs in response to endothelin-1 treatment (Fig. 3.4B). We found also that overexpression of lipin-1 in *fld* MEFs did not cause a decrease in PA accumulation compared to overexpression of a vector control (Fig. 3.5A). However, there was a significant increase in PA accumulation in response to endothelin-1 for *fld* MEFs overexpressing lipin-1 or vector control (Fig. 3.5A).

The translocation of PAP activity to membranes in response to PLD stimulation suggests that the lipins may play a role in PLD-mediated signalling (Fig. 3.6A,E,G-H). This work provides initial evidence from western blotting

analysis of both overexpressed and endogenous lipin-1 and lipin-2 in membrane fractions which does not support the translocation to membranes of lipin-1 or lipin-2 in response to treatment with PLD agonists (Fig.3.7A-B and Fig.3.9A-B). A possible explanation is that these overexpression of lipin-1 was relied on and endogenous lipin-1 may in fact translocate to membranes in response to PLD activation (Fig. 3.7, Fig. 3.8). Moreover, total cellular PA mass may remain unchanged between *fld* and control MEFs but rather the molecular composition of PA that accumulates could be altered in *fld* compared to control heterozygous MEFs. Again the total mass of PA might not change but knockdown of lipin might cause a redistribution of PA to other membrane compartments. Studies with fluorescent PA biosensors suggest that PA accumulates mainly on the plasma membrane and at cell-cell junctions in response to PLD stimulation with PMA [205].

There was a notable lack of accumulation of DAG (Fig. 3.4B) following PLD activation. It is possible that DAG turnover is very rapid in MEFs and frequently DAG accumulation has not been reported without the use of chemical inhibitors of lipolysis which we did not use in these studies. The translocation experiments using western blotting or PAP activity show a surprisingly large amount of lipin-1 or PAP activity at the membrane in the control cells under basal conditions. This could be due to lipin translocation to compartments other than the plasma membrane such as the ER where lipins are involved in *de novo* synthesis of lipids. PA accumulation in *fld* compared to control MEFs was not observed, contrary to our expectation (Fig. 3.4A). However, the PA

concentration curve reaches a plateau at treatment concentrations between 5 nM and 10 nM of endothelin-1 (Fig. 3.4A).

It is interesting to note that lipin-1 mRNA levels are lower in breast cancer lines such as MCF-7 and MDA MB 231 cells than in MCF-10A normal breast epithelial cells (Fig. 3.11A). Unlike PMA, endothelin-1 and LPA are physiological growth factors and endothelin-1 is secreted by MDA MB 231 breast cancer cells.

Cell migration is one of the effects of PLD signalling and we confirmed that endothelin-1 stimulated PLD-dependent migration (Fig. 3.12B). Our experimental data with means ± ranges showed that the endothelin-1 effect in stimulating cell migration was abrogated by pre-treatment of the cells with the primary alcohol butan-1-ol, but not by butan-2-ol (Fig. 3.12B). We tested for an effect of lipins on cell migration using the inhibitor BEL. We found that BEL alone caused a significant increase in cell migration compared to the increase promoted by MAFP alone (Fig. 3.12A).

Treatment of breast cancer cells with the pan-PLD inhibitor and isoform-specific inhibitors caused a 75% decrease in PA accumulation detected in labelling experiments, indicating that most of the PA accumulation due to endothelin-1 or LPA stimulation is due to PLD rather than through the hydrolysis of PIP_2 to DAG and IP_3 by PLC and subsequent phosphorylation of DAG to PA by DGK [47] (Fig. 3.10A). These findings are consistent with the work of others which shows that although activation of PLD by EGF stimulation does increase levels of DAG, inhibition of PA formation by addition of ethanol to EGF-treated

cells did not prevent the rise in DAG [225]. This suggests that the PA accumulation caused by the activation of PLD may not be a significant source of DAG and this is in agreement with the results obtained from the labelling studies in this work (Fig.3.4, Fig. 3.10A-B). In addition, PLC may be the main enzyme that catalyzes the formation of DAG in response to treatment with growth factors. The conversion of DAG to PA was inhibited with R59949, the most commonly employed and more selective pharmacological agent that inhibits Ca^{2+}-dependent DAG isoforms. R59949 specifically blocks the catalytic activity of Ca^{2+}-dependent DGK isoforms by binding to their catalytic domain [258]. Moreover, MEFs express at least six DGK isoforms and the lack of increase in DAG levels could reflect high activity of DGK enzymes [47]. Treatment of cells with LPA and R59949 caused decreased PA accumulation in lipid labeling experiments (Fig. 3.10A). The observation that PA accumulation in these cells shows a large component that is dependent on DGK activity is consistent with the finding that DAG does not show an endothelin-1-dependent or LPA-dependent increase as the concentrations of these agonists are increased (Fig.3.10A-B). This result could be explained if DAG turnover by DGK enzymes or DAG lipases were rapid. Similarly, previous work showed that in HEK293 cells, treatment with the dual PLD1/2 inhibitor, FIPI, decreased total cellular basal PA levels by 30% [174]. The increase in PAP activity associated with membranes in response to PLD activation supports a role for lipins in regulating PLD-dependent siganlling. PLD agonists promoted the accumulation of PA on membranes and this effect was partially abrogated by both PLD1 and PLD2 chemical inhibitors in MCF-7 cells (Fig. 3.10A). On the other hand, subcellular

fractionation could be required to isolate or enrich the pool of lipin on the plasma membrane.

Knockdown of lipins was critical to this project because there are no specific chemical inhibitors of lipins. The mechanism of action of BEL, the proposed inhibitor of lipins and $cPLA_2$, has been analyzed by mass spectrometry and shown to involve alkylation of thiol groups of cysteine residues of enzymes and it has been shown to inhibit glutathione-*S*-transferase [243]. BEL also does not inhibit LPP activity [243]. We obtained earlier results showing a stimulatory effect of BEL but not from MAFP, a $cPLA_2$-specific inhibitor, on LPA-induced migration of Rat2 fibroblasts (Fig. 3.12A). These results need to be validated by testing the effect of a lipin knockdown on agonist-mediated cell migration. However, treatment with BEL alone did not cause a significant increase in PA accumulation (Fig. 3.12C) and the mechanism of the stimulatory effect of BEL on cell migration is unclear.

The increased PAP activity detected in the membrane fraction (Fig. 3.6A,E,H) in response to PLD agonists could correspond to the translocation of endogenous lipin-1 to membranes, as seen from preliminary evidence from western blot analysis (Fig. 3.6G). In contrast, overexpressed recombinant lipin-1 did not translocate to membranes (Fig. 3.7A-B). We observed from the results of two separate western blots which showed superposition of anti-HA antibody with anti-lipin-1 antibody that lipin-1β did not translocate to membranes in response to endothelin-1 treatment (Fig. 3.7A-B). Preliminary evidence of a PLD-dependent increase in membrane association of PAP enzymes was observed when PAP

activity was measured whereas the translocation of lipin-1 analyzed by western blotting, was not significant (Fig. 3.7A-B). One reason for this may be the translocation of lipin-2 or lipin-3 to membranes in response to PLD agonists, which has not yet been studied. Similarly, the majority of endogenous lipin-1 is reported to be found in the cytosol [173] and the majority of overexpressed recombinant lipin-1 was found in the cytosol (Fig. 3.C-D). This is consistent with our western blot analysis from one experiment showing no lipin-1 in the membrane fraction without stimulation of PLD activity or stimulation with oleate (Fig. 3.6G). We determined that overexpression of lipin-1β in *fld* MEFs did not promote decreased phosphorylation of ERK1/2 in the membrane fraction at basal levels or in response to PLD activation (Fig. 3.8). This result was consistent with results comparing ERK1/2 activation in *fld* and control hearts [210].

Lipins promote the activation of intracellular GIVA PLA_2 through activation of PKC [238]. Activation of GIVA PLA_2 by agonists is also sensitive to inhibition of PLD activity [238,256]. Furthermore, treatment of differentiated U937 macrophages with ethanol, an inhibitor of PLD signalling, prevented the fMLP-stimulated increase in DAG levels and the release of arachidonic acid [238]. The upregulation of COX-2 also required PAP activity in U937 macrophages [237]. Increased expression of COX-2 in response to activation of TLR-4 by LPS was dependent on NF-κB activation [256]. In the TLR-4 signalling pathway, PKC can promote upregulation of COX-2 expression through NF-κB by activating MAPK and phosphorylating I-kB [256]. Activation of COX-2 was found to promote cell invasion in Dox-resistant compared to Dox-control cells. COX-2 overexpression

also promoted increased resistance to Doxorubicin in MCF-7 cells and correlated with increased genomic instability [251]. LPS treatment caused PA hydrolysis to decrease to a plateau at about 2 min treatment and consistently DAG levels peaked at 2 min of treatment [237]. Furthermore, addition of DAG to U937 macrophages treated with LPS increased COX-2 expression and the release of Prostaglandin E2 [237]. Increased DAG levels could promote upregulation of signalling pathways required for Dox resistance because the DAG kinase chemical inhibitor R59949 inhibited the activation of NF-κB and the phosphorylation of Bcl-2 [240]. Bcl-2 can be phosphorylated by PKCα at Ser-70 and this modification protected cells against apoptosis [259]. Treatment of MTLn3 mammary adenocarcinoma cells overexpressing Bcl-2 with a selective inhibitor of PKCα increased apoptosis [260].

Given the role the lipins in upregulating COX-2 expression, we sought to determine whether changes in lipin expression could be observed in Dox-resistant compared to Dox control cells [237]. Lipin-1 accounts for much of the Mg^{2+}-dependent PAP specific activity in cells [155]. We observed from RT-PCR experiments in which means ± ranges were measured that lipin-1 mRNA expression levels were higher in Dox-resistant cells than in Dox-control cells or in MCF-7 cells (Fig. 3.11A). Consistently, we found that PA levels were lower in LPA-treated Dox-resistant cells and there was a trend for higher DAG levels in Dox-resistant cells at basal levels and also after 1-2 min. of stimulation with LPA although the values were not statistically significant (Fig. 3.11E-F). However, we did not observe changes in the magnitude of PAP activity in Dox-resistant

compared to Dox-control cells (Fig. 3.11D). We also showed that LPA treatment after 2.5 hours induced COX-2 expression in *fld* and control MEFs (Fig. 3.11G). Moreover, there was no difference in DAG accumulation as promoted by PLD agonists between Dox-resistant and Dox-control cells. However, lipin-1 mRNA levels measured in one experiment increased in Dox-resistant compared to syngenic control and MCF-7 cells (Fig. 3.11A). We also found initial evidence that NF-κB expression is reduced in *fld* MEFs (Fig. 3.11H) and this suggested that lipin-1 could promote expression of NF-κB. Furthermore, phosphorylation of the p65 subunit of NF-κB promotes the expression of COX-2 [261].

We observed that in MCF-7 cells the DAG spike decreases rapidly and correspondingly the PA levels remain elevated and this could be due to high activity of DGK in these cells as demonstrated by the significant effect of R59949 on the LPA-stimulated increase in PA in MCF-7 cells (Fig. 3.11E-F). Previous work has shown that Raf-1 activation increased resistance of cells to doxorubicin whereas Akt activation had no effect [262]. We found that doxorubicin-resistant cells showed decreased PA accumulation and apart from one significant point there was no difference in DAG accumulation between Dox-resistant and Dox-control cells (Fig. 3.11F). However, the PAP activity of Dox-resistant compared to Dox-control cells did not appear to differ greatly in magnitude although the values represent means ± ranges and statistical analysis could not be performed. (Fig. 3.11D). Endothelin-1 promoted a more potent mitogenic effect by causing increased PA accumulation in syngenic control MCF-7 cells compared to Dox-resistant cells (Fig. 3.11E). It seems likely that changes in PLD activity or

membrane lipid synthesis could result from Dox-resistance rather than being a causative factor. Changes in phospholipid composition of membranes can alter membrane structure and are associated with the resistance of cells to the cytotoxicity of chemotherapeutic agents [263]. It would be informative to measure PA accumulation in Dox-resistant and Dox control cells using mass spectrometry. A further informative experiment would be to knockdown or overexpress lipin-1 in Dox-resistant cells and to determine whether lipin-1 expression could regulate Dox resistance by its role in converting PA to DAG. The relatively low lipin-1 and lipin-2 expression in Dox-resistant cells as compared to MCF-10A cells (Fig. 3.11A-B) would support a regulatory role for the lipins in mediating Dox-resistance but further experiments are required to investigate a possible role for lipins in Dox resistance. However, lipin-1 mRNA expression levels were lower in MCF-7 control cells than in Dox-resistant MCF-7 cells, whereas lipin-2 and lipin-3 mRNA expression levels were comparable in magnitude (Fig. 3.11A-C). This finding demonstrates the complexity of the role of individual lipin isoforms across various cell types.

CHAPTER 4: INSIGHTS INTO INTERACTIONS OF LIPINS WITH THE EPIDERMAL GROWTH FACTOR RECEPTOR

4.1. Introduction

The EGFR can be activated at the plasma membrane by the binding of EGF or the EGFR can be internalized and activated within endosomes to promote endosomal signaling. Endosomal EGFR can promote signaling through PI3-K and Akt [264]. The internalized EGFR-EGF complexes may also signal through associated proteins Grb2, Cbl, SHC, and GAP to promote cell survival and inhibit apoptosis resulting from serum withdrawal [264]. Many studies have identified proteins that associated in a complex with the EGFR at the plasma membrane in response to EGF ligand binding [90,227]. In contrast, proteins that interact with the EGFR at the plasma membrane in the absence of EGF stimulation have not been fully identified [96]. Activation of the EGFR in the absence of ligand is a major cause of the development of chemoresistance in breast cancer cells [96].

Experimental results presented in the previous Chapter showed that because of redundant function between the lipin isoforms, a genetic approach using lipin-1 depleted cells did not allow us to determine whether the lipins could be attenuating or promoting PLD signalling. The next approach used was to directly examine whether lipin-1 could interact with the EGFR. Jiang *et al* reported that PAP activity associates with the EGFR in the absence of ligand but this interaction is dissociated upon binding of the EGF [223]. Further evidence predicting that lipin-1 could interact with the non-activated EGFR came from our results in the previous Chapter showing increased PAP activity associated with membranes in response to PLD activation in cancer cells (Fig. 3.6A,E,H) and by

a previous study which showed that PAP activity co-immunoprecipitated with the EGFR [223].

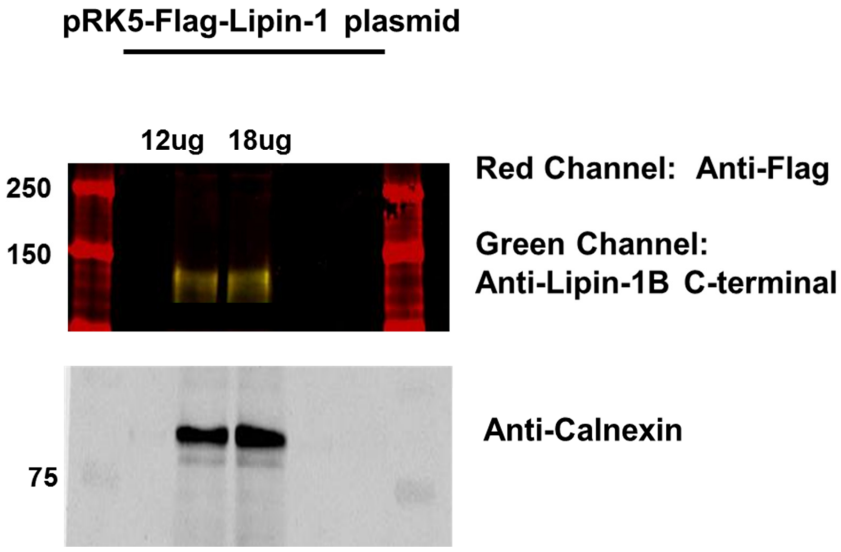
The goal of this Chapter was to examine the reported interaction of Mg^{2+}-dependent PAP activity with the EGFR. We predicted that lipin-1 or lipin-2 would co-immunoprecipitate with the EGFR in cells with high EGFR expression. We reasoned that treatment with EGF would cause a decrease in co-immunoprecipitation of the EGFR with lipins which would correspond to internalization of the EGFR as described previously [223]. We investigated this possibility using biochemical methods including co-immunoprecipitations and PAP assay and we performed confocal microscopy in cells in which lipin-1β or lipin-2 was overexpressed. First we used biochemical methods to determine whether lipin-1 could bind in a complex with the EGFR.

4.2. Lipin-1 and lipin-2 do not co-immunoprecipitate with the epidermal growth factor receptor in A431 cancer cells

Mg^{2+}-dependent PAP activity has been reported to co-immunoprecipitate with the EGFR. Given the emerging role of lipins in regulating signalling, we tested whether lipin-1 or lipin-2 would co-immunoprecipitate with the EGFR. MDA MB 231 cells express high endogenous levels of the EGFR (Fig. 4.1C). Our investigations on interactions between lipin-1 and the EGFR in cell lysates using immunoprecipitation with an anti-HA antibody and analyzed by western blotting did not result in a significant immunoprecipitation of EGFR with lipin-1 (Fig. 4.1D).

Co-IP experiments were performed in MDA MB 231 cancer cell lines, which highly expressed the EGFR (Fig. 4.1C). MDA MB 231 cells were transfected with flag-tagged lipin-1β or flag-tagged lipin-2 plasmid both of which were highly overexpressed in these cells as confirmed by western blotting (Fig.4.1A-B). Lysates from cells overexpressing lipin-1β showed a band detected by the anti-flag antibody that was superimposed with the band detected by the lipin-1 antibody (Fig. 4.1A). Similarly, overexpression of lipin-2 was confirmed by western blotting with anti-flag and anti-lipin-2 antibodies (Fig. 4.1B). We performed Co-IP of lipin-1 with anti-flag antibody but did not detect pulldown of the EGFR in the western blot analysis of the immunoprecipitate (Fig. 4.1D). Because the inability to detect EGFR in IP experiments in which lipin-1 was detected could be due to using the wrong conditions for the formation of the complex or the inability to solubilize the EGFR, we optimized several parameters of the CoIP including detergent concentration and the concentration of BSA used to preclear the sample (Fig. 4.1E). However, we were not able to see a substantial CoIP of lipin-1 and the EGFR under the optimized conditions (Fig. 4.1E). Many of the less stringent conditions used where the EGFR was detected also caused the EGFR to be detected in the absence of immunoprecipitation of flag-tagged lipin-1, indicating a non-specific binding of the EGFR with the anti-flag antibody used for IP (Fig. 4.1E).

A)


pRK5-Flag-Lipin-1 plasmid
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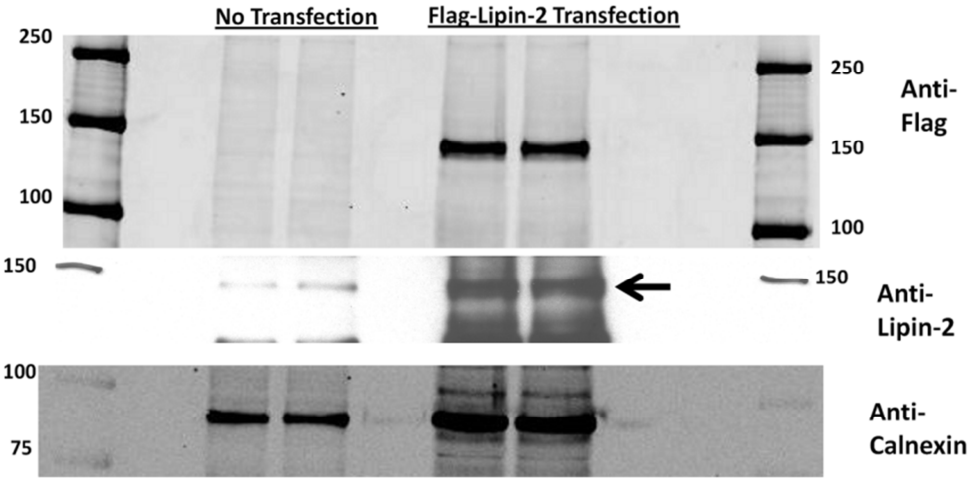
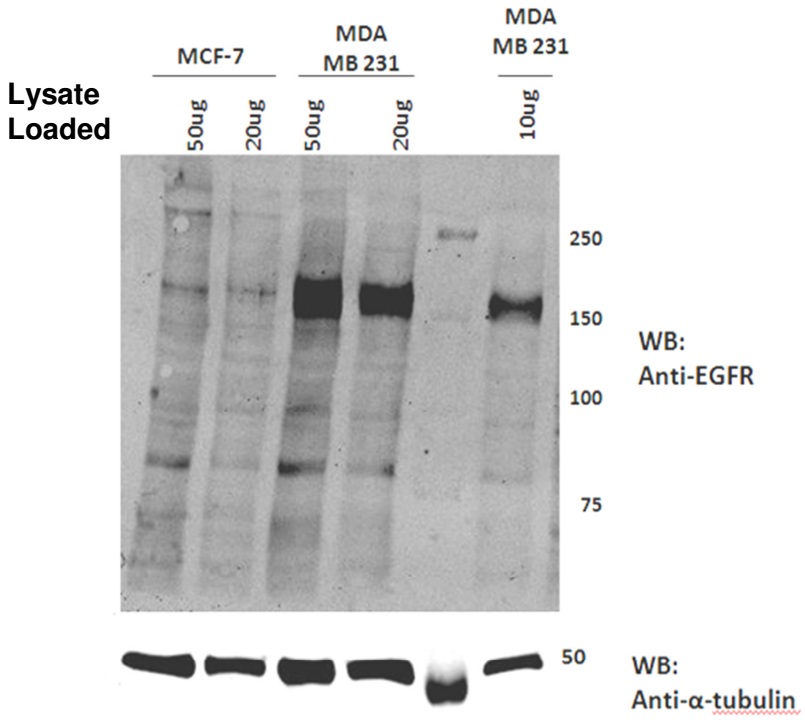
250
150
Red Channel: Anti-Flag
Green Channel:
Anti-Lipin-1B C-terminal
75
Anti-Calnexin

B)


No Transfection
Flag-Lipin-2 Transfection
250
150
100
250
150
100
Anti-
Flag
150
150
Anti-
Lipin-2
100
75
Anti-
Calnexin

C)


MCF-7
MDA MB 231
MDA MB 231
Lysate Loaded
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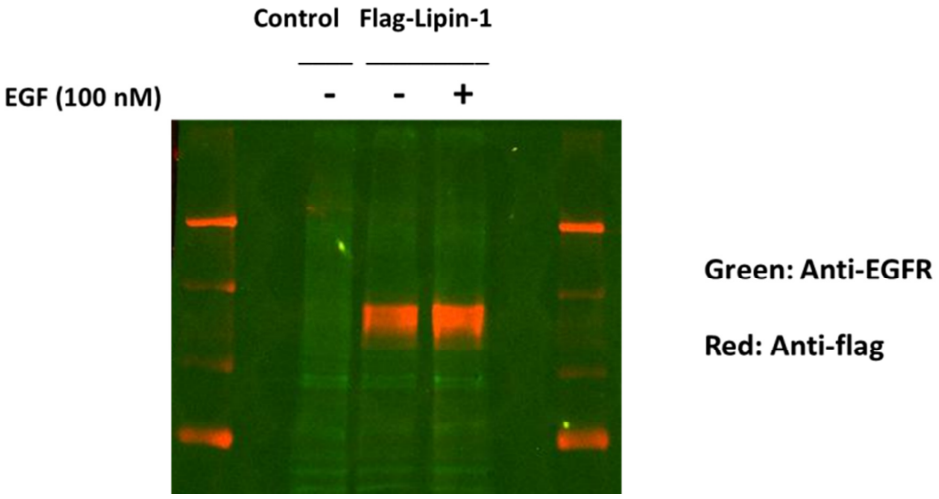
20ug
50ug
20ug
10ug
250
150
100
75
50
WB: Anti-EGFR
WB: Anti-α-tubulin

D)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Flag-Lipin-1
EGF (100 nM)
-
-
+
Green: Anti-EGFR
Red: Anti-flag

E)

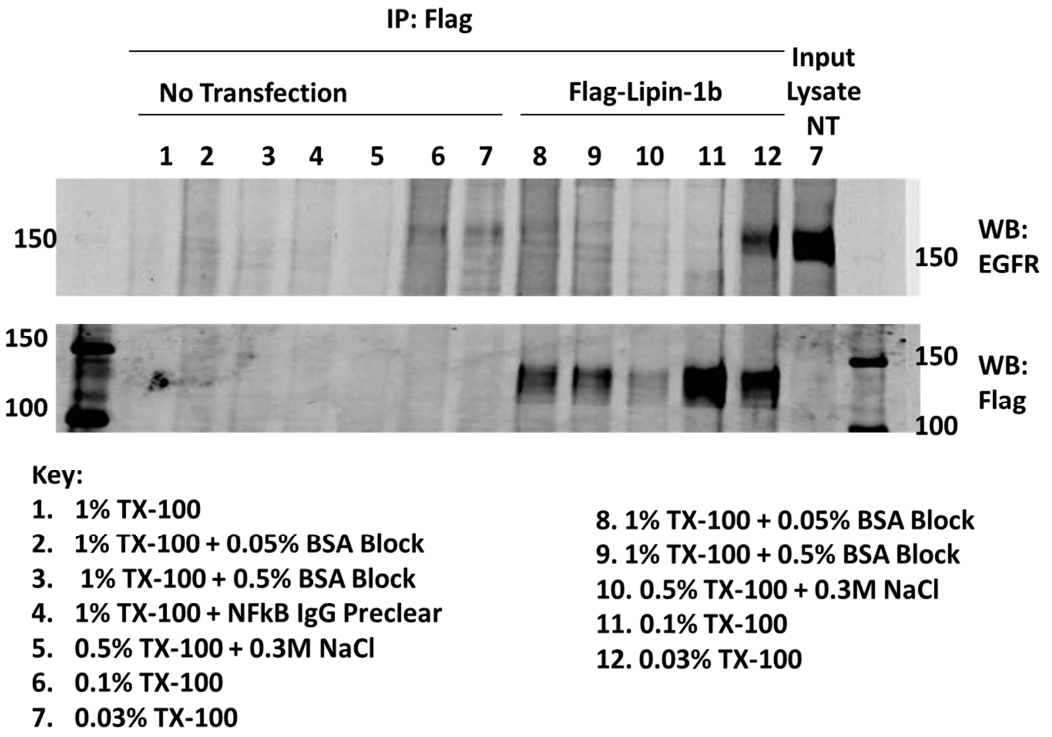


Figure 4.1. Lipin-1 does not co-immunoprecipitate with the EGFR to a significant extent. All experiments were done in MDA MB 231 cells except where indicated. A. Western blot analysis of overexpression of flag-tagged lipin-1β following transfection with flag-lipin-1β plasmid. This blot shows the superposition of the lipin-1 C-terminus-specific antibody (green fluorophore-tagged) with the anti-flag antibody (red fluorophore-tagged). **B. Western blot analysis showing overexpression of flag-tagged lipin-2 following transfection with flag-lipin-2 plasmid as analyzed by western blotting with lipin-2-specific and anti-flag antibodies.** For A and B, western blots are representative of 3 independent experiments. **C. Expression of the EGFR in MCF-7 and MDA MB 231 cancer cell lines as analyzed by western blotting with an antibody specific to the C-terminus of the EGFR.** Blot is representative of two independent experiments. **D. Co-IP in lysates overexpressing lipin-1β was performed with anti-flag antibody and the Co-IP was analyzed by western blotting with anti-flag antibody (red fluorophore) and anti-EGFR antibody (green fluorophore). E. Optimization of conditions for the attempted co-IP of the EGFR with HA-lipin-1.** Each number in the key (legend) refers to the corresponding lane on the blot. Triton-X-100 detergent (TX-100) concentration was varied as indicated. Blocking with BSA was performed for some samples (Lanes 2,3,8,9) and the percentage of BSA used is indicated. 0.3M NaCl treatment was used to decrease non-specific binding to the beads used in co-IP where indicated. The effect of preclearing the sample with an antibody against NF-kB was also tested (Lane 4). The blot is representative of results from two independent experiments.

4.3. Chemical crosslinking does not promote co-immunoprecipitation of lipin-1 with the epidermal growth factor receptor

When we could not detect Co-IP of lipin-1 with the EGFR in MDA MB 231 cells, we tested whether incubating cells with the cell permeable chemical crosslinkers paraformaldehyde (PFA) or dithio(bis)succinimidyl propionate (DSP) could promote the co-immunoprecipitation of lipin-1 with the EGFR (Fig. 4.2). DSP has a crosslinking arm distance of 12 Å and crosslinks amines. PFA crosslinks amide residues that are approximately 2 Å apart. By treating cell cultures with these crosslinkers, we attempted to crosslink residues of the EGFR or lipin, which could be close or far apart in order to attempt to preserve the hypothesized interaction of lipin with the EGFR, which could occur during transient signalling events. We found that neither PFA nor DSP treatment promoted increased co-immunoprecipitation of lipin-1 with the EGFR (Fig. 4.2).

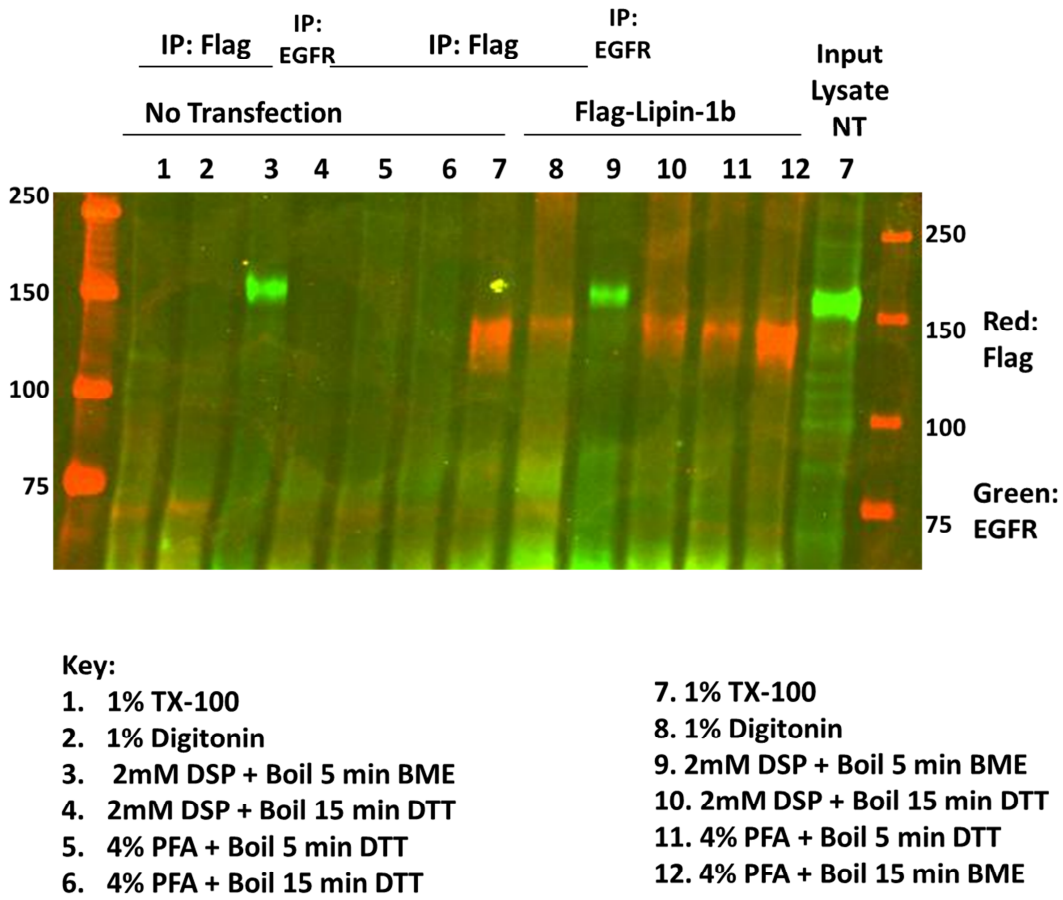
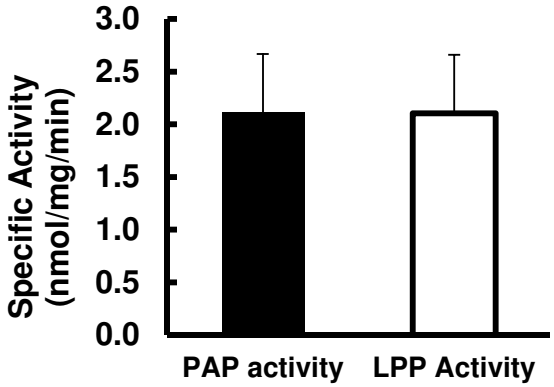


Figure 4.2. Crosslinking does not promote co-immunoprecipitation of lipin-1 with the EGFR A. Co-IP experiment in which IP was performed with an anti-EGFR antibody or an anti-flag antibody as indicated in A431 cells transfected with plasmid for flag-tagged lipin-1β. The result is representative of two independent experiments. The detergent Triton-X-100 (TX-100) was tested in lanes 1 and 7. β-mercaptoethanol (BME) and dithiothreitol (DTT) were tested for their ability to give the best reduction of bonds in the crosslinked complexes and dissociation of dimeric forms of lipin-1. Details about the crosslinkers used, DSP and PFA, can be found in the accompanying text. The mild detergent digitonin was also tested for its ability to preserve complex formation. The IP was boiled for the period of time indicated to determine the effect of the boiling time on disrupting crosslinked bonds.

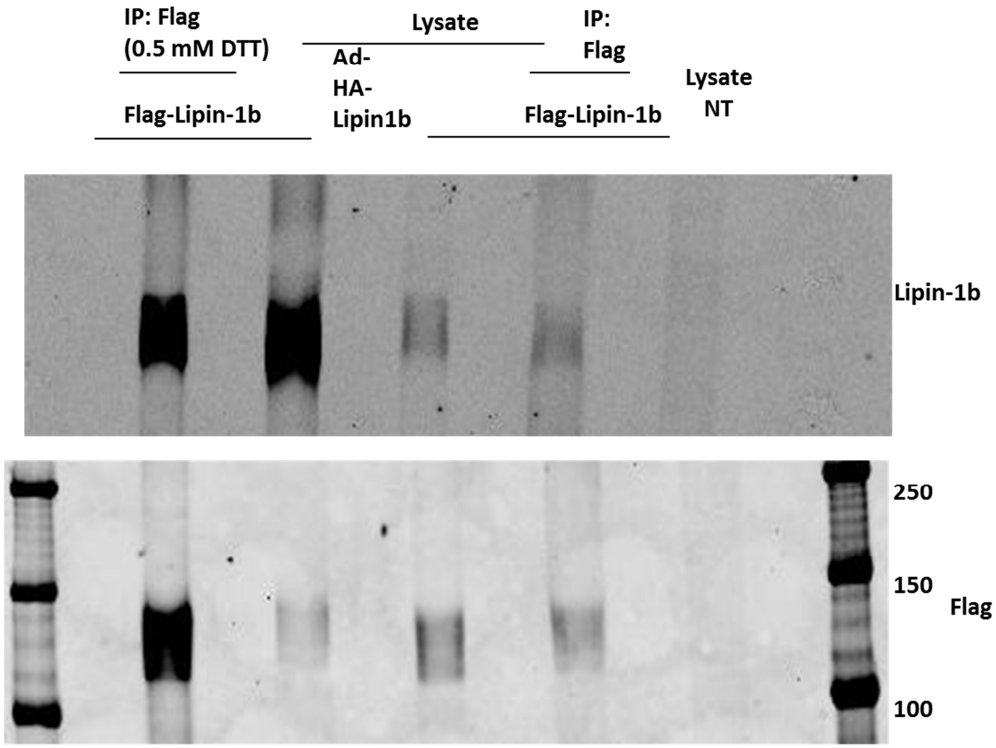
4.4. The phosphatidic acid phosphatase activity detected in epidermal growth factor receptor immunoprecipitates is largely due to lipid phosphate phosphatase activity

Contrary to earlier reports which found PAP activity but not LPP activity in A431 cells, LPP activity was detected in these cells (Fig. 4.3A). We demonstrated that immunoprecipitation with an antibody against HA can successfully immunoprecipitate HA-lipin-1 in the presence of 0.5 mM dithiothreitol (DTT) (Fig. 4.3B). Previous work showed that DTT was required for full activity of lipins and this work suggested that lipins could be co-immunoprecipitated in a catalytically active form (Fig. 4.3B). PAP activity was detected in a co-immunoprecipitation with an antibody against the C-terminus of the EGFR but a large percentage of this activity was from Mg^{2+}-independent activity due to LPPs (Fig. 4.3C-D). We also provided controls showing that lipin-1 or lipin-2 was successfully overexpressed in these experiments as indicated by increased PAP activity measurements or western blots showing successful overexpression (Fig. 4.3D).

A)


Specific Activity
(nmol/mg/min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PAP activity
LPP Activity

B)


IP: Flag
(0.5 mM DTT)
Flag-Lipin-1b
Lysate
Ad-
HA-
Lipin1b
IP:
Flag
Flag-Lipin-1b
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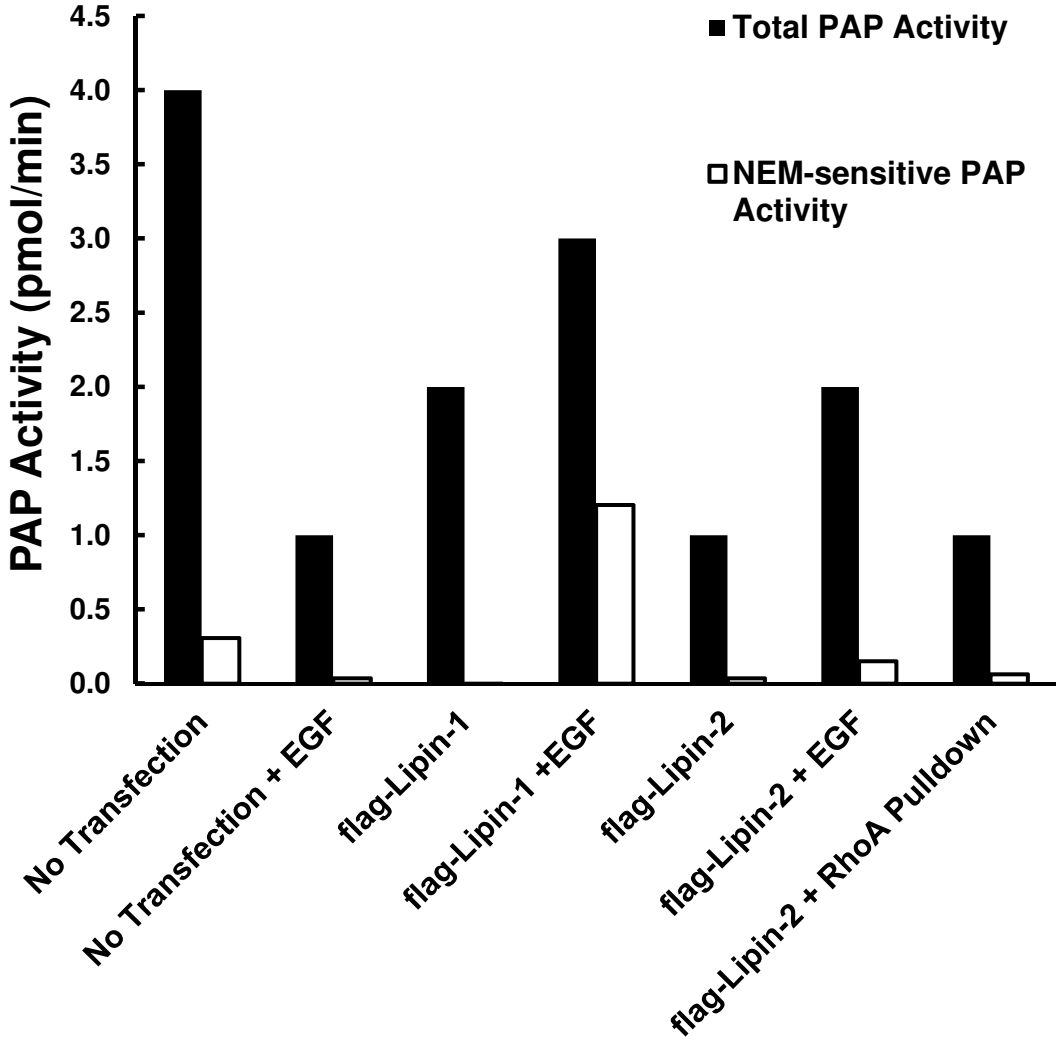
NT
Lipin-1b
250
150
100
Flag

C)


Total PAP Activity
NEM-sensitive PAP Activity
PAP Activity (pmol/min)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
No Transfection
No Transfection + EGF
flag-Lipin-1
flag-Lipin-1 +EGF
flag-Lipin-2
flag-Lipin-2 + EGF
flag-Lipin-2 + RhoA Pulldown

D)

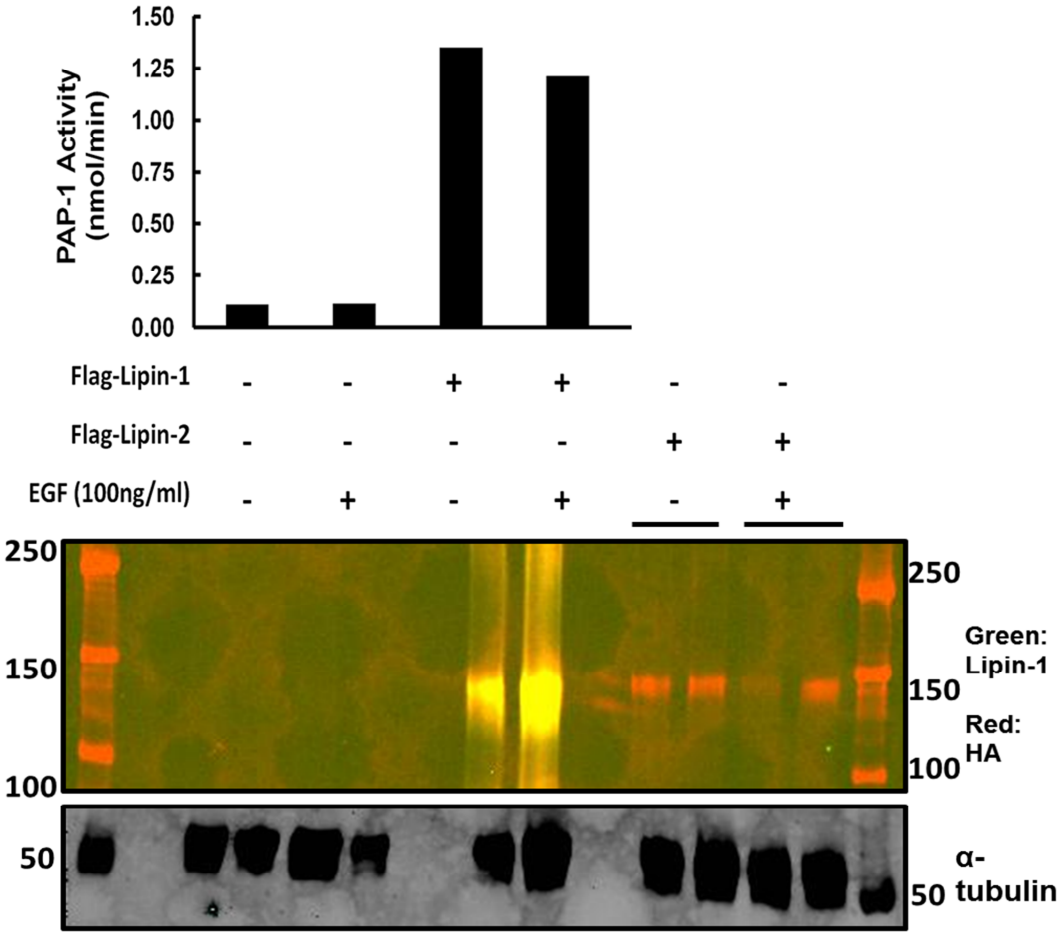

PAP-1 Activity (nmol/min)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Flag-Lipin-1 - - + + - -
Flag-Lipin-2 - - - - + +
EGF (100ng/ml) - + - + - +
250
150
100
50
Green: Lipin-1
Red: HA
α-tubulin

Figure 4.3. PAP and LPP activity is detected in immunoprecipitates in A431 cells. Cells in A and B were transfected with plasmid for flag-tagged lipin-1β. Cells in C and D were infected with adenovirus for HA-tagged lipin-1β. In all experiments, cells were with treated with 100 ng/ml of EGF or vehicle for 5 min. **A. PAP and LPP activity in cells without treatment of cells with agonist. Values represent means ± S.D. from 3 independent observations per treatment.** The means were not significantly different. **B. Immunoprecipitation in total cell lysates in the presence of 0.5 mM DTT is successful in immunoprecipitating lipin-1 with the anti-flag antibody.** A result from one experiment in which lipin-1 was detected with an anti-HA antibody and an antibody against the C-terminus of lipin-1. **C. PAP activity in EGFR immunoprecipitates. Cells were lysed with digitonin and the membrane fractions were collected and used for immunoprecipitation. D. Overexpression of lipin was verified by measurement of PAP activity and western blot analysis in the cytosolic fraction from cells lysed with digitonin from one experiment.** The anti-HA antibody was detected in the red channel and the anti-lipin-1 antibody, which is specific to the C-terminus of lipin-1 was detected in the green channel. The superposition of the two channels is depicted as the yellow bands.

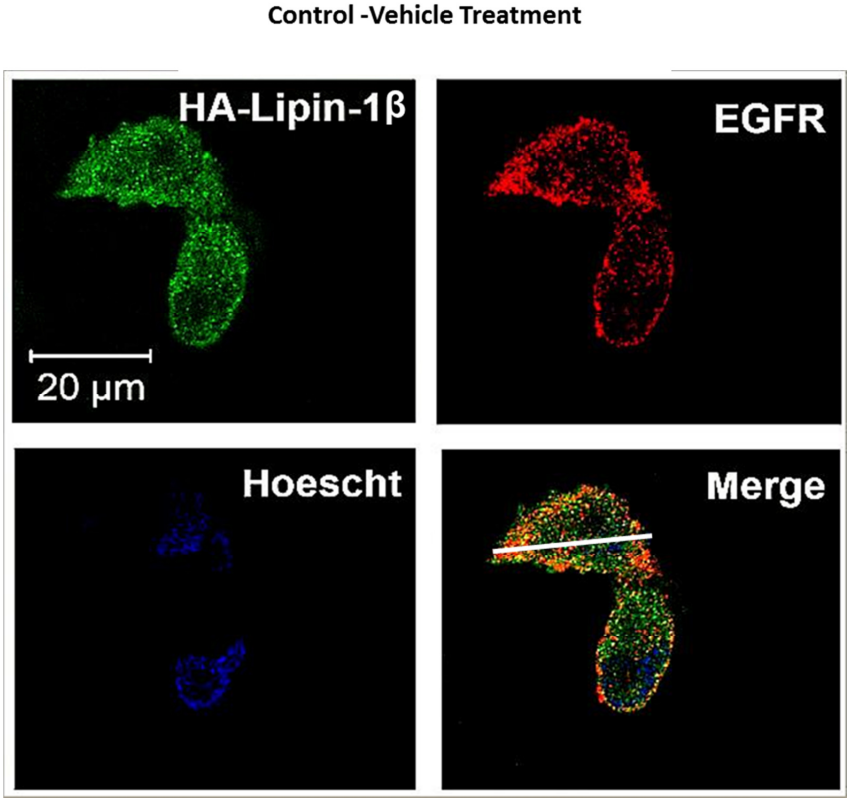
4.5. Preliminary evidence from confocal microscopy shows that lipin-1 localizes to the cell periphery in the presence of phospholipase D agonist

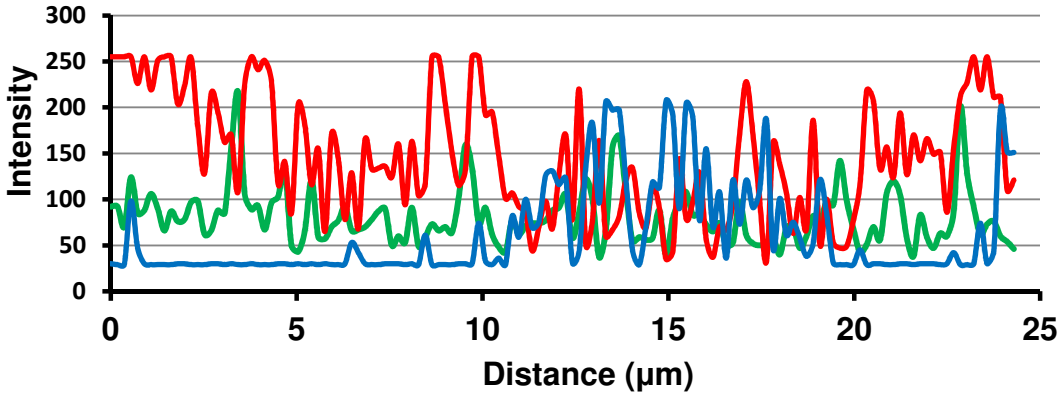
Endothelin-1 promotes paracrine and autocrine signalling in cancer cells [108]. It was also shown that endothelin-1 treatment caused transactivation of the EGFR in cancer cells [265]. We therefore sought to determine whether we could see evidence of lipin-1 on the plasma membrane and whether or not lipin-1 could co-localize with the EGFR. An antibody to the C-terminus of the EGFR was used to determine the localization of the EGFR in fixed cells. This EGFR antibody has been used for confocal imaging in previous studies [266].

Immunofluorescence of lipin-1β showed a staining distributed that was present throughout the cell, but largely absent from the nucleus. Staining of lipin-1 was strong at the perinuclear region and also in projections of cells (Fig. 4.4). Hoechst staining was used to select stained cells for analysis, which did not contain fragmented nuclei which indicate apoptosis of cells. Fragmented nuclei were observed in some cells. We analyzed a slice through the confocal image by drawing a line through the cell. The intensity along the line was evaluated from the histogram distribution with Image J software. The appearance of yellow color suggesting colocalization of lipin-1 and EGFR in the cells was analyzed along the distance of a line. Consistent with our expectation, the staining patterns of lipin-1β and the EGFR did not colocalize to a significant extent in the ER and nucleus as determined by an analysis of the overlap of pixel intensity along the distance of a line (Fig. 4.4A-B). We found that whether cells were treated with vehicle or with endothelin-1, the peaks of fluorescence for EGFR staining did not coincide with the peaks of fluorescence for HA-lipin-1β in the nucleus or cytoplasm (Fig. 4.4). However, we observed some localization of lipin-1 at the periphery of the cell, which supports a possible co-localization of lipin-1 with the EGFR (Fig. 4.4A-D). The endothelin-1- treated cells did not show significant colocalization of the staining (Fig. 4.4A,4.4E) since the peaks from the fluorescence intensity were not coincident to a significant extent. HA-tagged lipin-1 staining showed high intensity at cell projections in endothelin-1-treated cells and this is consistent with the localization of lipin-1 at the plasma membrane (Fig. 4.4B). This effect was also seen in the vehicle-treated cells in which the staining of HA-lipin-1 exhibited high intensity at regions at the edge of the cell representing the plasma

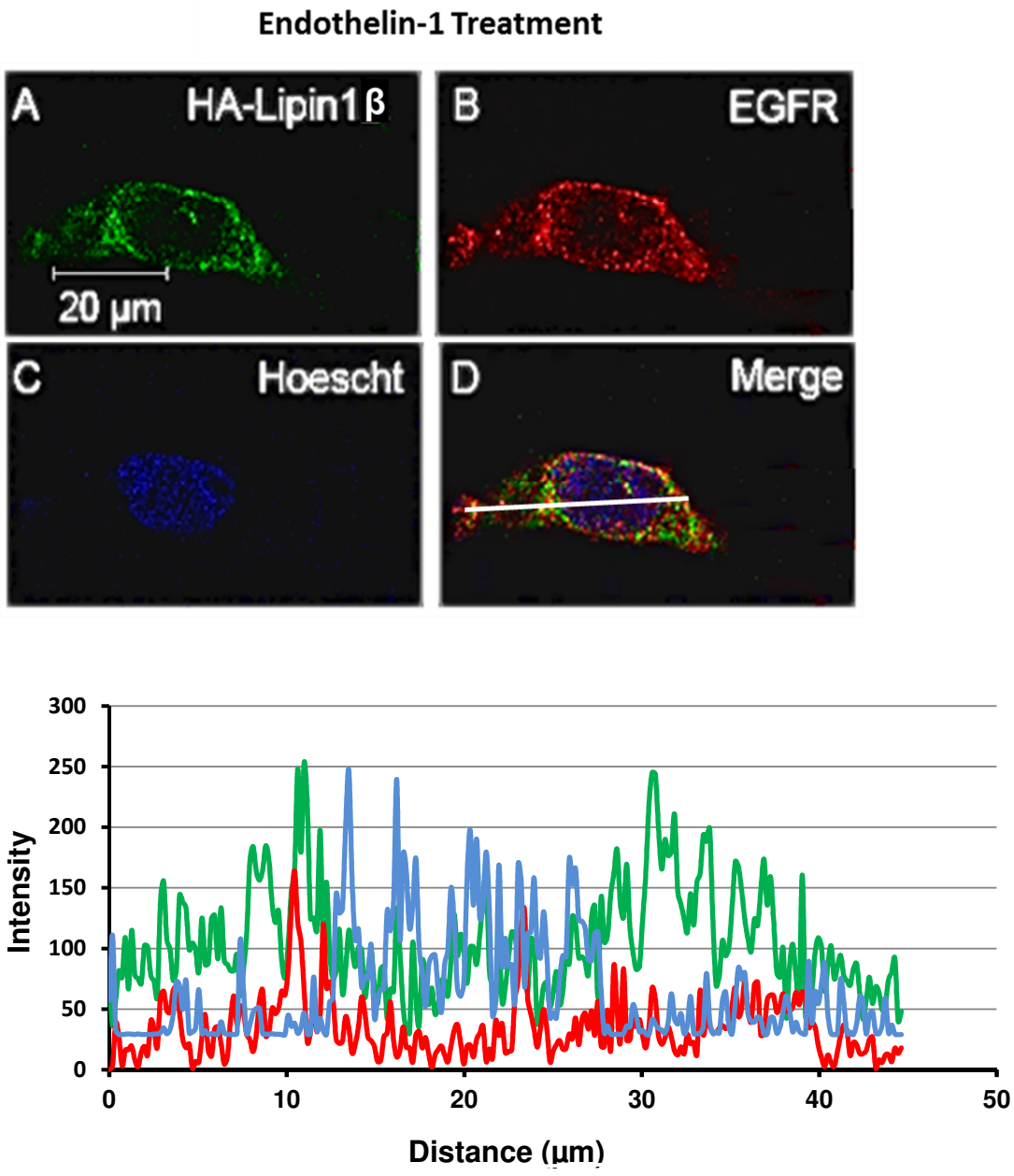
membrane and in these cells there was some colocalization of high intensity peaks of EGFR staining with high intensity peaks of HA-lipin-1 staining (Fig. 4.4A). In contrast, the cells infected with control adenoviral vector did not exhibit a high intensity of staining at the plasma membrane or a large colocalization with the EGFR (Fig. 4.4D). Thus, these results provide evidence that lipin-1 does localize to the plasma membrane.

A)

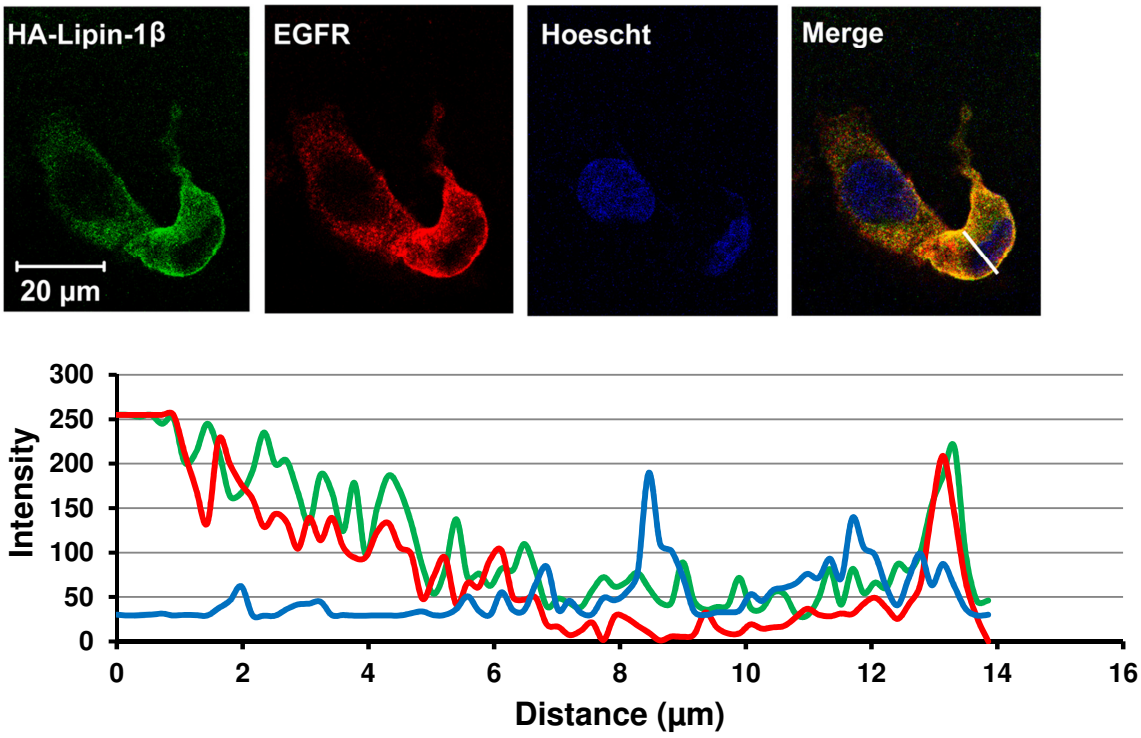

Control -Vehicle Treatment
HA-Lipin-1β
EGFR
20 μm
Hoescht
Merge


Intensity
300
250
200
150
100
50
0
0
5
10
15
20
25
Distance (μm)

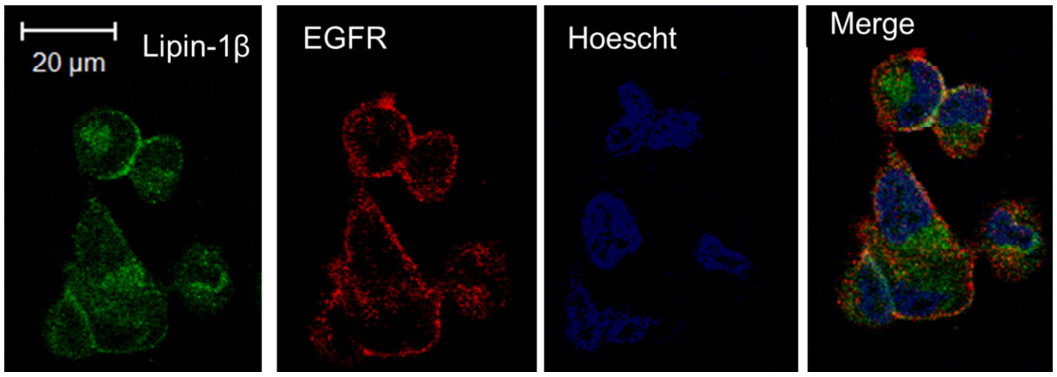
B)


Endothelin-1 Treatment
A
HA-Lipin1β
20 μm
B
EGFR
C
Hoescht
D
Merge
Intensity
300
250
200
150
100
50
0
0
10
20
30
40
50
Distance (μm)

C)


HA-Lipin-1β
EGFR
Hoescht
Merge
20 μm
Intensity
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
Distance (μm)

D)


20 μm
Lipin-1β
EGFR
Hoescht
Merge

E)

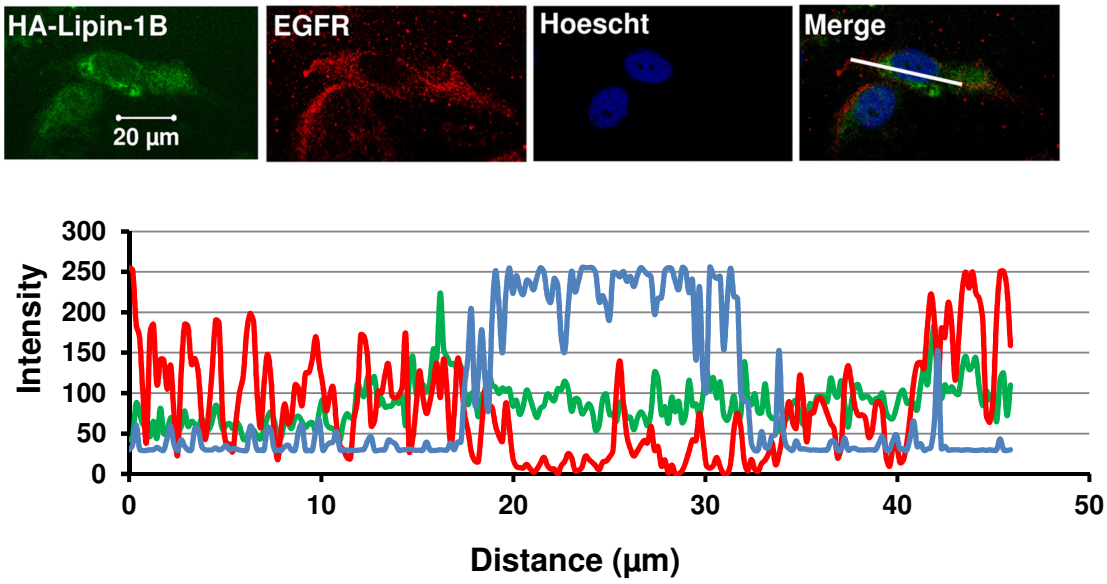


Figure 4.4. Preliminary evidence that lipin-1β localizes to the cell periphery in the presence of phospholipase D agonist. MDA MB 231 cells were infected with lipin-1β adenovirus or vector control adenovirus and treated with vehicle or endothelin-1 followed by fixing. Cells were stained with antibodies against the HA tag or against EGFR. Hoescht was used to stain nuclei as labelled in each panel. The last panel in each set depicts the overlay of the images from the green, red and blue channels. **A. Control cells infected with lipin-1β adenovirus and treated with vehicle for 5 min. B,C. Cells were infected with lipin-1β adenovirus and treated with 50 nM endothelin-1 for 5 min.** The arrow indicates a cell projection. **D. Cells were infected with lipin-1β adenovirus.** Cells were treated with endothelin-1. **E. Cells were infected with HA-lipin-1β adenoviral vector and were not treated with endothelin-1.** For each image for which colocalization was analyzed, a line was drawn through the superimposed images as indicated and the fluorescence intensity was determined along the line using Image J and plotted as a histogram. Images shown are representative of at least 2 separate images collected.

4.6 Discussion

From work in the previous Chapter, we observed both PLD-dependent translocation of PAP activity (Fig. 3.7A-B) as well as lipin-1 and lipin-2 present on membranes under basal conditions (Fig. 3.10A-B). These observations supported the possibility that lipins might co-immunoprecipitate with the EGFR in the absence of EGFR ligand.

We have found that A431 cells contain LPP activity (Fig. 4.4A). The report that A431 cells lack LPP activity could be explained by the use of a less sensitive assay for measurement of LPP activity by Jiang *et al* than the one employed in this work [223]. It is also known that long-term treatment with EGF upregulates the expression of LPP3, but does not affect the expression of LPP1 [129]. We determined that a large percentage of PA phosphatase activity in EGFR immunoprecipitates is due to LPP activity (Fig. 4.4C). This could have occurred because of non-specific pulldown of the membrane components which could be enriched in LPP since LPP is an integral membrane protein. Emerging data have shown that in cancer cells the EGFR localizes predominantly in caveolae in the absence of ligand [267]. Binding of ligand causes the activated dimeric forms of the EGFR to localize at bulk plasma membrane and then rapidly move to cell-surface clathrin-coated pits from where endocytosis of the EGFR can occur [267]. However, we detected the EGFR in the positive control membrane fraction cell lysate solubilized in co-immunoprecipitation buffer and used for our attempted co-immunoprecipitation of the EGFR with lipin-1 that was loaded on the Lane 7 labelled 'Input lysate NT' (Fig. 4.2). Detection of EGFR in the

membrane fraction lysate indicated that solubilization of the EGFR was achieved with the method used in this work (Fig.4.2).

Previous studies in HEK293 cells in which single-molecule imaging and immunoelectron microscopy were performed showed that in the absence of ligand the EGFR is localized at the plasma membrane in various monomeric, dimeric, and oligomeric forms [91]. Furthermore, in OVCA 433 human ovarian carcinoma cells, endothelin-1 treatment for 5 min. caused phosphorylation of the EGFR and phosphorylation of Shc [265]. The confocal images in this Chapter show that the yellow areas of colocalization of the EGFR with lipin-1β are clearly not in the nucleus nor in the ER, although the EGFR reportedly localizes to the nucleus [229]. Some colocalization of the EGFR with lipin-1 is observed by visual inspection at a 3 μm distance and at a 24 μm distance along the analyzed line drawn through the cell and these distances correspond to regions at the periphery of the cell (Fig.4.5A). The yellow regions could represent the plasma membrane, actin or lamellipodia, which would support a role for lipin-1 in regulating cell migration (Fig.4.3A-B). However, there is not much overlap between the pixel intensity for the lipin-1 staining and EGFR staining indicated from the colocalization analysis. In particular, the EGFR and lipin-1 appear to co-localize very poorly in cells treated with endothelin-1 (Fig. 4.5B-C) and we have not found strong evidence to support the hypothesis that endothelin-1 might promote the translocation of lipin-1 to membranes where it could bind to the EGFR. In contrast, the sparse co-localization that is observed at the cell periphery is observed in the vehicle-treated control and this would be consistent

with an interaction of lipin-1 with the EGFR at the membrane that could be disrupted by PLD stimulation (Fig. 4.3A-B). Treatment of cells with endothelin-1 transactivates the EGFR and causes ERK1/2 activation whereas treatment with AG1478, a chemical inhibitor of EGFR activation, abrogates the effect of endothelin-1 on EGFR activation [268]. Therefore we could expect lipins to dissociate from the EGFR in response to endothelin-1 treatment but to remain membrane-bound and associated with other signalling proteins such as PKCε [223]. We would expect to see a decrease in colocalization of the EGFR and lipin-1 staining in the cell in response to endothelin-1 stimulation although lipin-1 should remain at the membrane. In cells that were unstimulated with endothelin-1, colocalization of the fluorescent signal from the EGFR with lipin-1 is noted (Fig. 4.5A). When the cells were stimulated with endothelin-1, we observed dissociation of the EGFR and lipin-1 and decreased overlap in the fluorescence intensity as indicated by poor coincidence of the peaks (Fig. 4.5B). Furthermore, it is likely that once the EGFR dissociates from lipin that the EGFR would be internalized by endocytosis [90,267]. We could not clearly observe evidence for internalization of the EGFR. The interaction between EGFR and lipin-1 could be indirect. However, we could not find compelling evidence that lipin-1 binds in a complex in which EGFR is also bound (Fig. 4.2D-F, Fig. 4.3-4.5). These results are consistent with findings from studies of the EGFR interactome, which did not detect lipins in microarray analysis of co-immunoprecipitates of the EGFR [93,227]. Furthermore, confocal imaging studies performed in cancer cell lines with concanavalin A as a plasma membrane marker showed that it is mainly the unstimulated EGFR that colocalizes with concanavalin A at the plasma

membrane [269,270]. Treatment of cells with EGF causes the endocytosis of the EGFR [90] and also was reported to disrupt the interaction of PAP activity with the EGFR which could occur when the EGFR is internalized [223]. These studies would need to be repeated with markers for the ER and Golgi as well in order to track endocytosis of the EGFR. Previous studies in fixed HeLa cells showed that HA-tagged lipin-1 was predominantly localized in the cytosol and in live cells GFP-tagged lipin-1 showed a similar distribution [173]. Staining for HA-tagged lipin-2 was observed in the cytosol but also associated with the ER and the nuclear envelope in fixed HeLa cells [173]. Consistently, we found that lipin-1-HA showed a diffuse distribution throughout the cytosol, which also appeared to be perinuclear (Fig. 4.5A-D) but this would need to be confirmed by staining of the cells with a nuclear envelope marker and performing a colocalization analysis.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1. General Discussion

We hypothesized that the lipins metabolize the PA produced as a second messenger by PLD. Initial work was done using the available pharmacological inhibitors to determine the relative contribution of PLD1/2 and DGKs to PA accumulation in breast cancer cells and in MEFs. Work done in breast cancer cell lines supported the hypothesis that PA accumulates in response to PLD activation and that a significant proportion of PA accumulation is due to both PLD1 and PLD2 activation in these cell lines. This work is subject to the limitations and non-specific effects of pharmacological inhibitors and future work will use other models to examine these findings. Our finding that NF-κB expression is reduced in *fld* MEFs suggests that lipin may upregulate the transcription of NF-κB in MEFs. Contrary to our hypothesis on the effect of PA in Dox-resistant breast cancer cells, PA accumulated less in Dox-resistant cells than in syngenic control cells. This finding highlights the complexity of PA signalling and suggests that in Dox resistance, signalling pathways other than PA signalling and SK-1 activation might also be involved.

One of the major mechanisms of chemoresistance in breast cancer cells is mediated by COX-2 upregulation. Another major mechanism of acquired resistance to chemotherapy in breast cancer cells occurs through ligand-independent activation of the EGFR. We found that lipins did not appear to affect activation of the EGFR in the absence of ligand, contrary to previous reports that indicated PAP activity interacts in a complex with the EGFR. Furthermore, we did

not find compelling evidence for lipins to bind to the EGFR. Taken together, these results from co-immunoprecipitation and confocal microscopy have not found compelling evidence for involvement of lipin-1 or lipin-2 in interacting with the EGFR. Future studies that will likely utilize other antibodies and staining techniques requiring confocal microscopy and possibly live imaging studies could uncover a role of lipins in attenuating or promoting signalling effects of PLD.

However, there did appear to be a potential role for lipins in regulating chemoresistance in Dox-resistant cells and lipin-1 expression was increased relative to Dox-control cells. We also found that NF-κB expression is downregulated in MEFs depleted of lipin-1 expression. Consistently, we also found that PAP activity translocates to membranes in response to stimulation with the PLD agonists LPA and endothelin-1 in MDA MB 231 breast cancer cells. These results warrant further investigation into dual roles for the lipins in breast cancer cells as PAP enzymes increasing DAG levels and as transcriptional coactivators regulating NF-κB expression.

5.2. Summary and Conclusions

This work represents an investigation of whether lipin-1 or lipin-2 can dephosphorylate the PA produced by PLD activation and thereby attenuate or activate the PLD signalling pathway. Work from one experiment provided initial

evidence that lipin-1 overexpression does not attenuate ERK1/2 phosphorylation downstream of PLD activation in the membrane fraction from *fld* MEFs. However, we found initial evidence of a translocation of PAP activity to membranes in response to treatment of cells with endothelin-1 and LPA as PLD agonists. The accumulation of PA was followed by the accumulation of DAG and was time-dependent in cancer cells. These studies thus provide initial evidence supporting future work on the involvement of lipin-1 in the PLD pathway. However, no changes in PA labelling were seen in *fld* MEFs as compared to control MEFs expressing lipin-1 and this finding was consistent with our other work showing PAP activity in *fld* MEFs and indicating a redundant PAP activity of lipin isoforms in MEFs. Overall, the presence of PAP activity due lipin-2 and lipin-3 in our *fld* MEFs and breast cancer cell models probably prevented us from determining definitively whether lipin-1 is a major regulator of the pools of PA on the membrane that are involved in PLD-mediated formation of PA and signalling.

Although we showed that PAP activity translocates to membranes in a PLD-dependent manner, we could not show directly that PLD-derived PA is regulated by the PAP activity of lipins. We found indirect evidence supporting a role for lipins in regulating PLD-dependent signalling from the translocation of PAP activity to membranes in response to PLD activation. We could not show that the combined PAP activity of the lipins regulates PA or DAG in MEFs because ablation of lipin-1 did not significantly decrease PAP activity in MEFs. Moreover, overexpression of lipin-1 or lipin-2 did not significantly affect PA or DAG levels in our experiments. However, these findings could be explained by

the presence of excess lipin that is able to translocate to membranes. Several lines of evidence have shown that basal PA levels increase in response to lipin-1 knockdown in adipose tissue [190], in *fld* hearts [210] and in response to PAH1 knockdown in yeast [271], but these studies did not measure PLD-derived PA.

This work is the first reported to examine the reported binding of lipin-1 to the EGFR using coimmunoprecipitation and confocal microscopy. We performed these studies to follow up on the translocation of PAP activity to the membrane in response to PLD agonists which we detected. However, association of lipin-1 or lipin-2 with the EGFR was not detected to a significant extent in two different cancer cell lines based on co-immunoprecipitation experiments. After optimization of detergent conditions for co-immunoprecipitation (Fig. 4.2E, 4.3), our co-immunoprecipitation technique was specific as demonstrated by the fact that co-immunoprecipitation with anti-flag antibody did not reveal the presence of the EGFR despite high enrichment of the EGFR in the membrane fractions produced by digitonin lysis. However, the finding that LPP activity is co-immunoprecipitated with the EGFR is likely due to the fact that the EGFR is enriched in caveolae [267] and both LPP2 and LPP3 have been detected in caveolae by immunostaining [60]. We found PAP activity in the co-immunoprecipitates using anti-EGFR antibody (Fig. 4.4C) and this is consistent with results, which found that treatment of cells with the inhibitor of PAP activity inhibitor, propranolol, caused increased internalization of the EGFR [89]. Furthermore, preliminary results from confocal imaging did not show colocalization of lipin-1 and the EGFR (Fig.4.5A-B).

The hypothesis that lipin-1 or lipin-2 plays a major role in regulating the PLD pathway has not been conclusively supported by these studies. However, much work from the literature has reported changes in PLD signalling effects including activation of MEK and ERK1/2 via phosphorylation in *fld* peripheral nerve tissue and we have shown that PLD activation causes translocation of PAP activity to membranes (Fig.3.7A-B, Fig3.9). More work is needed to reveal the complex relationships between PA accumulation and PLD signalling outcomes such as ERK1/2 activation, SK-1 translocation, and to understand the relationship between Dox resistance and a possible role of PAP activity in regulating PLD signalling that could promote Dox resistance.

More work is needed to demonstrate the effect of lipins on PA synthesized through the PLD pathway and its signalling effects in breast cancer cells. Recent work in the field has shown that PLD expression is upregulated in breast cancer and appears to be a central mediator of breast cancer progression through inhibition of degradation of the Myc onco-protein and through signalling effects through Sos and Raf kinase which can increase cell migration [10,17]. PLD activity has also been studied as a potential therapeutic target in treatment of breast cancer [79,106]. This work demonstrates that lipin-1 might not be a significant regulator of PLD-mediated PA accumulation in some cell types, especially those expressing all three lipin isoforms. However, PAP activity does translocate to membranes in response to PLD-dependent upregulation of PA whereas evidence against the translocation of lipin-2 in response to PLD

agonists was found. Work from *fld* and control MEFs suggests that the PAP activity of the individual lipin-1 isoform at the plasma membrane is not sufficient to abrogate PLD-mediated PA accumulation. However, work in which lipin-1 or lipin-2 was overexpressed in MEFs and Rat2 fibroblasts did show a trend for increased PA accumulation indicating that high PAP activity does not completely account for conversion of PA to DAG. This finding is consistent with a previous report of a lack of effect on PA accumulation in cells ovexpressing catalytically inactive mutants of lipin-1β as compared to wild type lipin-1β after labelling with [^{3}H]-palmitate in HepG2 cells [174]. This finding also lends support for a major role for DGK in PA-dependent signalling pathways. Studies of the regulation of the PLD signalling pathway in drug-resistant breast cancer cells also showed a time dependent PA accumulation at 2 min and showed that DAG increased at 5 min of LPA stimulation when there was a corresponding decrease in PA, implying the activity of a PAP enzyme that responds to PLD activation.

5.3. Future Directions

5.3.1. The role of PAP activity in ERK1/2 signalling

This work raises several questions about the role of lipins in PLD-mediated signalling. First, it is yet unknown whether the PAP activity or the transcriptional function of lipins or both functions are required for lipin-1 to prevent upregulation of ERK1/2 activation and this could be determined by the time frame of treatment

with agonist. Studies could be done initially by transfecting *fld* MEFs with plasmids encoding catalytically inactive lipin-1 mutants to determine whether ERK1/2 upregulation can take place in the presence of mutant lipin-1 lacking PAP activity. These experiments could be done by treating cells with PLD agonists on a timescale on the order of minutes to determine whether activation of PLD and subsequent translocation of lipin-1 could occur in order to activate ERK1/2. These studies could also be done over several hours in order to detect a mechanism of ERK1/2 upregulation requiring transcriptional upregulation of genes in the thus far unknown signalling pathway. In this pathway, PAP activity could activate NF-kB or other transcription factors through upregulation of DAG levels and PKC since it was shown that PAP activity was required for the effect on transcriptional upregulation of PPARγ in adipose tissue [190]. The function of lipin-1 to regulate transcription in the nucleus could also be required for signalling. It will be important to demonstrate whether the effects of lipins on ERK1/2 activation require PAP activity. This could be done by measuring ERK1/2 activation in WT and *fld* MEFs transfected with plasmids for WT lipin-1 or with catalytically inactive mutants.

5.3.2. The role of lipins in regulating NF-κB expression

The effect of lipin-1 mRNA expression on NF-κB expression should be verified in MEFs by RT-PCR. If this is a significant effect of lipin-1 as a transcriptional activator, it could explain why the PAP activity in Dox-resistant and

Dox-control cells was not different. The effect of lipin-1 in affecting lipid signalling in Dox resistance could thus be independent of DAG levels and of PKC activation. A further step would be to study the binding of NF-κB using electrophoretic mobility shift assays in nuclear extracts from control and *fld* MEFs to look for differences in NF-κB activation. Chromatin immunoprecipitation (CHIP) assays would also be appropriate to determine which sequences of the NF-κB gene lipin-1 could bind to.

5.3.3. Studies on phospholipase D signalling in the peripheral nervous system of *fatty liver dystrophy* mice

Future studies of a role for lipins in PLD-mediated signalling will likely focus on genetic approaches in appropriate models, on cellular biology techniques, and on mass spectrometric analysis. The results from this work show that all three lipin isoforms are expressed in fibroblasts and in many cancer cell lines including breast cancer, fibrosarcoma and also in non-cancerous cell lines such as fibroblasts. Recent studies have revealed that PA accumulates in adipose tissue and peripheral nerve tissue when lipin-1 is depleted, and changes in TAG levels in macrophages are also observed [6,233,235]. Future studies of a role for lipins in the PLD pathway will likely focus on these tissues. The redundancy between lipin isoforms demonstrated in this work and by others will make it difficult to demonstrate isoform-specific functions of lipins in cells derived from these tissues such as skin, breast, and fibroblast. Future work will require studying

each of the lipin isoforms individually and determination of which lipin isoforms are responsible for the growth factor-induced PA accumulation.

Future studies on a possible role of lipins in the PLD pathway will likely focus on cell and tissue models in which PA accumulation has already been reported. For instance, PA accumulation is physiologically relevant to and associated with demyelination in the *fld* mice. Studies may be performed in Schwann cells and brain tissue from *fld* and control mice to determine whether knockout of PLD could abrogate the PA accumulation to thus establish a link between PLD and a role for lipin signalling. Further studies might determine whether knockout of PLD in embryonic developing Schwann cells could compensate for the PA accumulation observed and allow myelination to proceed as normal in these animals. Further studies might pursue the possible effects of PLD knockout in tissues lacking or depleted in one or more of the lipin isoforms.

5.3.4. Studies on phospholipase D signalling in the Kennedy pathway and the role of lipins

If an accumulation of growth-factor stimulated PA is seen following knockdown of lipins but not in controls, a further direction of this work could be to determine whether PAP activity of lipins is required for the translocation SK-1 to membranes in response to PA accumulation. It will also be necessary to do a PLD isoform-specific knockdown with siRNA or to transfect the cells with catalytically inactive dominant/negative PLD mutants for each PLD isoform to

demonstrate which PLD isoforms are catalyzing formation of PA that is the substrate for lipins. Pharmacological studies could be done also to inhibit PLCβ or PLCγ to see whether PA accumulation in these cells is due to PLD or PLC. The PA could also accumulate though the Kennedy pathway. In order to differentiate between PA and DAG produced from glycerol-3-phosphate through the *de novo* synthesis pathway versus that produced through the PLD pathway, pre-treatment of the cells with triacsin C could be needed to determine the amount of PA and DAG labelled compared to controls. Triacsin C is an inhibitor of acyl CoA synthetases (ACS) isoforms ACS1 and ACS4 [272] but it is also a non-specific inhibitor. Furthermore, it would be of interest to determine whether molecular species of PA and DAG are predominantly produced in the PLD pathway in response to stimulation with PLD agonists. This would be useful since it is known that lipin-1 shows a substrate preference for medium unsaturated chain fatty acids in adipose tissue and without treatment with agonists [190]. Quantification of lipid species could be done with mass spectrometry.

5.3.5. Subcellular localization and a signalling role of lipins

Unlike LPA and other extracellular lipids, PA and DAG are intracellular lipids and are not shown to stimulate extracellular receptors nor to be secreted. In this work,we showed that PAP activity translocates to cellular membranes. We were unable to show an interaction of lipin-1 with the EGFR using co-

immunoprecipitation. One reason for this could be disruption and loss of lipid rafts during co-immunoprecipitation. Further studies on the interaction of lipins with the EGFR could probe for the lipid raft-specific marker caveolin-1 [112] to determine whether EGFR in lipid rafts is properly solubilized during the co-immunoprecipitation.

Future studies could focus on subcellular localization and quantification using specific fluorescent probes. This might represent an advantage over isolating the plasma membrane pool of lipins using gradient ultra-centrifugation. This might allow determination of the cause of the high background of lipins on membranes seen in cells in this work. Although fractionation with percoll gradient ultracentrifugation could also be done to isolate the plasma membranes from nuclear membranes, endosomes, and ER/Golgi membranes, this approach is often susceptible to contamination of fractions.

This work showed that lipin-1 localizes to the periphery of breast cancer cells. Confocal microscopy could be used to determine whether lipin translocates to the plasma membrane using the commonly used plasma membrane marker, concanavalin A [269]. A finding of colocalization of concanavalin A with lipin-1 would provide evidence that lipins are involved in signal transduction. Similarly, the localization of lipin-1 could be further examined with a stain for actin microtubules which would indicate whether lipin-1 might be localizing to microtubules. Attempts at detection of lipin translocation by western blotting have been hampered by higher than expected levels of lipin on membranes under basal conditions which increases baseline levels. New cell-permeable

probes for PA are becoming available that will allow determination of localization and binding partners via fluorescence resonance energy transfer (FRET). FRET would allow an initial detection of interactions of lipin with EGFR or other plasma membrane-localized proteins. These studies can be done within the cell and would be physiologically relevant. Very likely, sequestration in specific subcellular compartments or local accumulation of bioactive lipids could be an important method of signalling without affecting total bulk cellular PA or DAG mass. Schwann cells could also be a useful model in which to investigate interactions of lipins at the plasma membrane using FRET.

A third direction for future work could be to determine binding interactions of the lipin isoforms. The lipins may participate in signalling by hydrolyzing PA or they may also participate in association with other proteins at the membrane. Future studies could be done to overexpress tagged lipin-1 and perform co-immunoprecipitation. This type of experiment could be done for cells fractionated by digitonin lysis in order to identify interactions of lipins at the membranes versus in the cytosol. Binding partners identified by this type of analysis could be investigated with confocal microscopy in order to determine the physiological significance in signalling. Lipin-1 has been shown to bind to NFATc4, and binds to PGC-1α [231]. Lipin-1 also associates with 14-3-3 proteins in the cytosol in HEK293 cells [186]. Likely, such an analysis might identify interacting partners of membrane-associated lipin-1 such as the PLD isoforms, Sos, or Raf and provide definitive evidence for the involvement of lipins in PLD-mediated signal transduction.

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