Survival signal generated by phospholipase D

by

Li Hui

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

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ABSTRACT

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Advisor: Professor David A. Foster

A critical aspect of tumor progression is the generation of survival signals that overcome default apoptotic programs. Previous data suggested that PLD might contribute to tumorigenesis by overcoming the apoptotic signal induced by stress conditions such as serum deprivation or over-expression of an oncogene. Cellular response to stress is frequently mediated by p53. Therefore we examined the effect of PLD on p53. We found PLD suppresses the p53 response to DNA damage in cells where PLD has been shown to provide a survival signal. Elevated PLD also suppresses DNA damage-induced apoptosis.

The study was extended to human breast cancer cells where p53 is mutated and overexpressed. Surprisingly, the stability of mutant p53 is enhanced by PLD in two breast cancer cell lines-- MDA-MB231 and BT549. Also, mutant p53 is required for survival in breast cancer cells that have elevated PLD activity. Moreover, mutant p53 was required for migration and invasion in these two cell lines, implicating a cooperation of elevated PLD activity and gain of function mutation of p53 for survival and metastasis properties of human breast cancer cells.

Another aspect of transformation is suppression of protein phosphatase 2A (PP2A). Suppression of PP2A by SV40 small t-antigen has been reported to be critical for the transformation of human cells with SV40 early region genes. We therefore examined the effect of PLD on PP2A activity. Elevated PLD activity in the human breast cancer cell line MDA-MB-231 suppressed PP2A activity in an mTOR-dependent manner. Consistent with a critical role for PP2A in PLD survival signals, either SV40 small t-antigen or pharmacological suppression of PP2A restored survival signals lost by suppression of either PLD or mTOR.

The capability of PLD to suppress tumor suppressor p53 and PP2A implicate that PLD can accomplish much of what SV40 early antigen has accomplished. By suppressing wild type p53 and PP2A and promoting the oncogenic function of mutant p53, PLD provides survival signals that contribute to tumorigenesis.

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Thanks to all my colleagues and collaborators for making a very good research environment for me: Dr. Minhao Zhong, Dr. Desmond Jackson, Dr. Yuhong Chen, Dr. Troy Joseph, Dr. Lizhong Xu, Dongon Lyo, Yang Zheng, Alfredo Toschi, Rafal Pielak, Vanessa Rodrik, Noga Gadir, Ming shi, Avalon Garcia. Working in our lab has been a great experience for me, I learned a lot from all my colleagues and I enjoyed spending time with them.

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List of Abbreviations

eIF-4E: eukaryotic initiation factor 4E

4EBP1: eIF-4E binding protein-1

FRB domain: FKBP12-rapamycin complex binding domain

HDM2: human homologue of murine mdm2

MAPK: Mitogen-activated protein kinases

MDM2: transformed 3T3 cell double minute 2

mTOR: mammalian target of rapamycin

PA: Phosphatidic acid

PARP: Poly-(ADP-ribose) polymerase

PC: Phosphatidylcholine

PH domain: Pleckstrin homology domain

PLD: Phospholipase D

PI-4-P-5-K: Phosphatidylinositol 4-phosphate 5-kinase

PI3K: Phosphatidylinositol-3-kinase

PP2A: protein phosphatase 2A

S6K: Kinase of the S6 protein

siRNA: short interfering RNA

CHAPTER I

INTRODUCTION

Phospholipase D

Phospholipase D (PLD) is a phospholipid enzyme that catalyzes the hydrolysis of phosphatidylcholine and produce choline and phosphatidic acid (PA). PA is a critical second messenger that mediates the mitogenic function of PLD. Two isoforms of mammalian PLD genes that are approximately 50% identical have been reported (hPLD1 and hPLD2), (Colley *et al* 1997), hPLD1 has 1072 amino acids and a molecular mass of 124 kDa (Hammond *et al* 1995), hPLD2 has 932 amino acids and a molecular mass of 106kDa (Colley *et al* 1997). Various characteristics of PLD1 and 2 are presented in Fig 1.1.

Fig 1.1

CHARACTERISTICS	PLD1	PLD2
PKC/ARF/Rho Responsive	Yes	No
PIP2 Dependence	Yes	Yes
Molecular Weight kDa	~120	~106
Basal activity	Low	High
Substrate Specificity	PC	PC
Transphosphatidylation	Yes	Yes
Subcellular localization	PM,CEM,ES	PM,CEM

Fig1.1 Biochemical properties of phospholipase D1 and D2**.** Presented are various characteristics of PLD1 and PLD2, corresponding references are presented in the text. PM-plasma membrane; CEM-caveolae enriched membrane; ES-endosomes; PIP2-phosphatidylinositol-4, 5-bisphosphate.

PLD has been implicated in membrane trafficking, cytoskeletal reorganization, receptor endocytosis, exocytosis, and cell migration (Exton *et al* 2002). PLD has also been shown to be involved in cell proliferation. The activity of PLD is elevated in response to platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, and insulin-like growth factor 1 (Plevin 1991, Motoike 1993, Song 1994, Karnam 1997, Sa 1999 and Banno *et al* 2003). PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src, v-Ras, v-Fps, and v-Raf (Song 1991, Carnero 1994, Jiang 1994, Jiang 1995 and Frankel *et al* 1999). Thus, there is a growing body of evidence linking PLD activity with mitogenic signaling. Interestingly, elevated PLD has been shown to contribute to cell transformation and survival. It has been reported that elevated expression of either PLD1 or PLD2, in combination with overexpression of c-Src or EGF receptor, transforms rat fibroblasts (Lu 2000, Joseph *et al* 2001). Fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 were able to provide survival signals that prevented apoptosis (Zhong *et al* 2003). The capability of PLD to cooperate with a tyrosine kinase such as c-Src or EGFR to transform cells or provide survival signals would implicate PLD as a good candidate oncogene in cancer, especially those cancers that have elevated tyrosine kinase such as c-Src or EGFR.

As expected, elevated expression and activity of PLD is reported in several human cancer tissues including breast cancer (Uchida 1997, Noh *et al* 2000), gastric and renal cancers (Uchida 1999, Zhao *et al* 2000). Hence, it is of interest whether the elevated PLD in human cancer tissues plays any role in tumorigenesis. Our lab has shown that

blocking PLD activity by using either a catalytic inactive mutant of PLD or primary butanol will induce apoptosis in breast cancer cell line MDA-MB231(Zhong *et al* 2003) and renal cancer cells (Toschi *et al*, unpublished data) . These cells have a very high level of PLD activity, this finding implicates that PLD provides survival signaling in cancer cells.

There are several downstream targets of phosphatidic acid generated by PLD, the important two are Raf/MAPK and mTOR, which contribute to the mitogenic properties of PLD. Raf has a PA binding site in its COOH terminus (Ghosh *et al* 1996) and it has been proposed that the generation of PA facilitates the recruitment of Raf to the plasma membrane, where it can participate in activation of the mitogen-activated protein (MAPK) kinase pathway (Rizzo 1999, 2002, Ghosh *et al* 1996).

mTOR, the mammalian target of rapamycin, is another interesting downstream target of PLD. mTOR is a protein kinase that regulates both cell cycle progression and cell growth (Kuruvilla 1999, Schmelzle *et al* 2000). Four years ago, PA was shown to interact with mTOR competitively with rapamycin (Fang *et al* 2001). Blocking mTOR activity with rapamycin can induce apoptosis in MDA-MB231 cells in the absence of serum (Chen *et al* 2005), further implicating mTOR as a downstream target of PLD. mTOR phosphorylates S6-kinase and 4E-BP1 to enhance translation of most mRNA transcripts (Schmelzie 2000, Hay *et al* 2004). Another target of mTOR implicated in the regulation of protein translation is protein phosphatase 2A (PP2A) (Janssens *et al* 2001). PP2A is a major serine/threonine phosphatase in eukaryotic cells. It appears to be critically involved in cellular growth control and potentially in the development of cancer. Aberrant expression, mutation or deletion of PP2A subunits are involved in cellular

processes leading to tumor formation (Wera 1995, Wang 1998, Takagi 2000, Ruediger 2001, Koma *et al* 2004). PP2A dephosphorylates the mTOR substrates S6-kinase and 4E-BP1 (Peterson *et al* 1999). mTOR was reported to phosphorylate TAP42/alpha 4 protein, one of PP2A regulatory subunits and enable it to associate with PP2A catalytic subunit (Nanahoshi *et al* 1998). This association inhibits the phosphatase activity of PP2A-C toward recombinant 4E-BP1 phosphorylated by mTOR (Di Como *et al* 1996, Nanahoshi *et al* 1998), implicating the negative regulation of PP2A activity by mTOR.

PLD and Transformation

The ability of PLD to cooperate with elevated expression of a tyrosine kinase is reminiscent of a model for transformation of primary cells based on Weinberg's work (Land 1983, Dotto 1985, Hahn 1999 and Elenbaas *et al* 2001). In their model, two groups of genes are required for cell division, group I genes such as Ras, Src that stimulate "competence" and passage through an early G1 growth factor-dependent restriction point. They also stimulate exit from a quiescent G0 state induced by the absence of growth factors. Group II genes facilitate "progression" through cell cycle checkpoint and/or prevent apoptosis, such as Myc and SV40 large T antigen which overrides the gatekeeper function of p53 and Rb. Cooperation of these two groups can transform rodent primary cells as shown in Fig 1.2. In this oversimplified context, the ability of PLD to complement elevated expression of a tyrosine kinase, which activates Ras proteins, would suggest that PLD genes could be group 2 (gatekeeper inhibitors) genes that facilitate progression through cell cycle checkpoints and prevent apoptosis. This would indicate that elevated PLD activity is able to provide what T antigen accomplishes. T antigen sequesters and inactivates p53 and Rb (Pipas *et al* 2001), both of which block passage through cell cycle checkpoints. p53 also stimulate apoptosis when there is excessive damage to the cell. It would be one of the major tumor suppressors to overcome if a tumor is about to emerge. Actually, more than 50% of human cancer has p53 mutation. PLD has been shown to prevent apoptosis in cells with high intensity Raf signals or overexpression of c-Src (Joseph 2001, Zhong *et al* 2003). Thus the ability to

prevent apoptosis may be due in part to the ability to inhibit one or more of the tumor suppressors such as p53 or Rb.

Compared to rodent cells, the transformation of human primary cells requires more genetic alterations. Weinberg and colleagues demonstrated that human cells could be transformed with a combination of H-Ras, hTERT, SV40 Large T antigen, and SV40 small T antigen which inhibits PP2A (Hahn *et al* 1999, 2002). Thus it appears that inhibition of PP2A is a critical additional requirement for human cell transformation. Since PLD provides survival signals in human breast cancer cells and PP2A is a downstream target of mTOR, it is highly possible that suppression of PP2A mediate survival signals provided by PLD.

Fig1.2

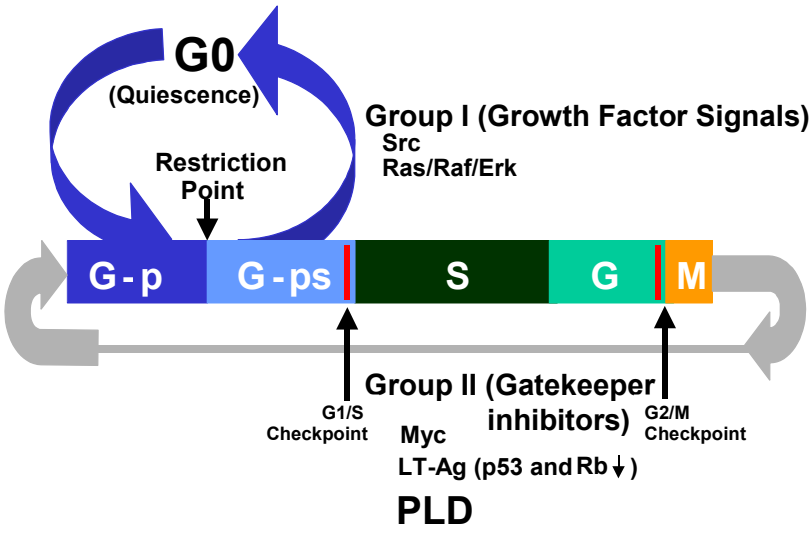


Fig1.2 PLD and transformation. Two complementation groups for cell transformation are required to regulate progression through different stages of the cell cycles. As shown, there are signals that facilitate passage through the restriction point or exit from a quiescent G0 state, and there are signals necessary for passage through cell cycle

checkpoints. PLD, like Myc and SV40 large T antigen, cooperates with Group I signals to transform rat fibroblasts. Transformation of human cells requires additional alterations that provide immortality, usually achieved by the expression of the telomerase gene.

p53

p53 is a tumor suppressor that functions as a transcription factor. It is involved in the regulation of cellular proliferation, cell cycle progression, DNA repair and apoptosis. Normal p53 protein is rapidly eliminated by virtue of its short half-life, in response to stress stimulus such as DNA damage or an oncogene activation , p53 is activated and stabilized through different mechanism and activate the transcription of genes important in cell cycle inhibition, apoptosis, genetic stability, and inhibition of angiogenesis (Vogelstein *et al* 2000).

Somatic TP53 gene alterations are frequent in most human cancers and inherited TP53 mutations predispose to a wide spectrum of early-on-set cancers, such as Li-Fraumeni Syndrome. Most TP53 mutations abrogate p53 transcriptional activity and result in the loss of anti-proliferative properties, however, some mutation of p53 result in gain of oncogenic functions including attenuating the function of the p73 gene or increased tumorigenesis. Most of the mutations in the p53 gene are missense, rather than truncation or deletion mutants (Hinds 1990, Hollstein 1994 and Hainaut *et al* 2000), and the missense mutations are clustered in DNA binding domain, in contrast, the post translational modification sites are not mutated in cancer. In cells that remains a wild type allele of p53, mutant p53 can act like a dominant oncogene through a "dominant negative" effect by interacting with and inactivating wild type p53 (Blagosklonny *et al* 2000). In cells that lack wild type allele, p53 mutants have been shown to confer tumorigenic properties (Ditter 1993, Hsiao 1994, Lanyi 1998, Scian *et al* 2004), indicating that the ability of p53 genes to contribute cell transformation goes beyond dominant negative effects. Moreover, tumor-derived p53 mutants have been shown to

activate promoters not activated by wild type p53 including those for the EGF receptor (Deb *et al* 1994), c-Myc (Frazier *et al* 1998), c-Fos (Preuss *et al* 2000), and others (Yang 1999, Van Oijen 2000 and Cadwell *et al* 2001).

In my thesis study, we found that elevated PLD activity is able to suppress wild type p53 expression as well as PP2A activity; both of them mediate the survival signals provided by PLD. The capability of PLD to suppress p53 and PP2A implicate that PLD can accomplish much of what SV40 early antigen has accomplished. Another very interesting phenomenon we found is that PLD is required for the stabilization of gain of function mutant p53 in two human breast cancer cell lines that has relatively high PLD activity. Given the high numbers of human cancer that has elevated PLD activity, clarifying the role of PLD in tumorigenesis will lead to strategies for targeting PLD in these cancers.

CHAPTER II

MATERIALS AND METHODS

Cells, Cell Culture, and Plasmids

3Y1 rat fibroblasts overexpressing c-Src (3Y1$^{c\text{-}Src}$ cells) were described previously (Ghosh, S *et al* 1996 and Hornia, A *et al* 1999)**,** the generation of 3Y1 and 3Y1$^{c\text{-}Src}$ cells that conditionally express PLD1 (3Y1-P1 and 3Y1$^{c\text{-}Src}$-P1 cells) was described previously (Joseph *et al* 2001). PLD1 expression and activity were induced with 10 μM ponasterone A (PonA) (Stratagene). MCF-7 and MDA-MB231 human breast cancer cells were obtained from the American Type Culture Collection. All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% bovine calf serum (HyClone). The MCF-7 cell lines stably expressing PLD2 were established by transfection with pcDNA3.1 (–)-hPLD2 by using Lipofectamine Plus reagent (GIBCO) according to the manufacturer's instructions. pcDNA3.1 (–)-hPLD2 was constructed as follows. The human PLD2 gene was excised from pBluescript-SK-hPLD2 (Colley, w *et al* 1997) with NotI and HindIII and was ligated into the polylinker region of the pcDNA3.1 (–) expression plasmid (Stratagene) which was cut with NotI and HindIII. The plasmid was amplified in *Escherichia coli* (XL-1-Blue host strain; Stratagene). The plasmid was then stably transfected into MCF-7 cells under G418 selection. The plasmid expression vectors for PLD2 (pCGN-mPLD2) and the dominant negative PLD2 mutant (pCGN-mPLD2- K758R) (Colley *et al* 1997) were from Dr. Michael Frohman (SUNY-Stony Brook). The SV40 small t-antigen expression vectors pCEP4/Smt and pCEP4/Smt (mut3) (Sontag *et al* 1993) were obtained from Estelle Sontag (Southwestern Medical School, Dallas). The MDA-MB231-P2DN cells are pooled clones of stable transfectants

described previously (Roderick *et al* 2005). Transfection was performed using Lipofectamine 2000 reagent (Gibco) according to the vendors instructions.

Materials

Generation of the monoclonal antibodies raised against p53 (pAb240 and pAb421) was described previously (Bargonetti *et al* 1993). Antibodies against Akt, phosphorylated Akt (Ser473), S6 kinase, phosphorylated S6 kinase (Thr389), mitogen-activated protein (MAP) kinase, phosphorylated MAP kinase (Thr202/Tyr204), and poly(ADP ribose) polymerase (PARP), BAD, phosphorylated BAD(Ser112), 4EBP1, mTOR were from Cell Signaling Technology. Antibodies against MDM2, HDM2, p21, PP2A, unmethylated PP2A, actin, and tubulin were from Santa Cruz Biotechnology. Antibodies to PLD1 and PLD2 were from Upstate Biotechnology. For nonimmune controls, we used ChromPure rabbit or mouse immunoglobulin G (IgG) from Jackson ImmunoResearch. Rapamycin and cycloheximide (CHX) were obtained from Sigma-Aldrich. U0126, PD98059, and LY294002 were obtained from Cell Signaling Technology. Camptothecin (CPT) and adriamycin (ADR), fostriecin, and cyclosporin A were obtained from Calbiochem. Rapamycin was obtained from LC Laboratories;

Western blot analysis

Samples were adjusted into gel-loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol) and then heated for 5 min at 100°C prior to separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After samples were transferred to nitrocellulose membranes (Osmonics), membrane filters were blocked with 5% nonfat dry milk in

phosphate-buffered saline (PBS) with 0.05% Tween 20 and then incubated with the appropriate antibody diluted in 5% nonfat dry milk in PBS with 0.05% Tween 20. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used, and the bands were visualized by using an enhanced chemiluminescence detection system (Pierce).

Assay of PLD activity

PLD activity was determined by a transphosphatidylation reaction in the presence of 0.8% butanol as described previously (Lu *et al* 2000). Cells in 100-mm culture dishes were prelabeled with [^{3}H]myristate for 4 to 5 h in DMEM containing 0.5% bovine serum. Lipids were extracted and characterized by thin-layer chromatography. Relative levels of PLD activity were then determined by measuring the intensity of the corresponding phosphatidylbutanol band in the autoradiograph with a Molecular Dynamics scanning densitometer and Image-Quant software.

Cell viability assays

Cell viability was determined by trypan blue exclusion. After various treatments, cells were collected, washed, and treated with trypan blue at a concentration of 0.4% (wt/vol). After 10 min, trypan blue uptake (dead cells) was determined by counting on a hemocytometer

Immunoprecipitation

Cells were washed twice with ice-cold PBS and scraped into the modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.6), 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM

EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 1x protease inhibitor cocktail, consisting of 0.5 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 1 μM leupeptin, 0.15 μM aprotinin, and 1 μM protease inhibitor E-64. The cells were then incubated at 4°C for 25 min by gentle rocking, sonicated for 20 s on ice, and centrifuged at 12,000 x *g* at 4°C for 10 min. The supernatant was precleared with protein G-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech), and 500 μg of the precleared proteins was adjusted to 500 μl in the modified RIPA buffer and then incubated with the antibody for 1 h as described above. The immunocomplex was captured by incubation with 50 μl of protein G-Sepharose 4 Fast Flow bead slurry and collected by centrifugation at 12,000 x *g* for 20 s at 4°C. The beads were washed three times with the modified RIPA buffer and once with wash buffer (50 mM Tris [pH 7.6]), and subjected to Western blot analysis.

PP2A activity

Cells were lysed on the plates with phosphatase lysis buffer containing 20 mM HEPES (pH 7.4), 10% glycerol, 0.1% Igepal CA-630, 1 mM EGTA, 30 mM b-mercaptoethanol, 1 mM PMSF, 1 μM leupeptin, 0.15 μM aprotinin. PP2A-C immunoprecipitation complexes were prepared as described above, except for the different lysis buffer used. Agarose beads were washed twice with phosphatase lysis buffer and once with phosphatase assay buffer (Promega), and the activity of PP2A was determined using a malachite green phosphatase assay protocol with a phosphopeptide (R-RA-pt-VA) as the substrate(Promega Madison, WI), followed by the measurement of absorbance at 620 nm, PP2A activity are given in picomoles phosphate per minute per microgram of protein. Data are reprentative of three experiments showing similar results.

siRNA

The mTOR siRNA duplexes were obtained from Cell Signaling Technology. A non-targeted negative control duplex siRNA was used as a negative control. Cells were plated on 6-well plates in medium containing 10% serum at 20% confluence. After one day, transfection of siRNA was performed using transfection reagent (Mirus) with 100 nM siRNA, and 3 days later cells were lysed and analyzed by Western blot analysis with mTOR antibodies.

CHAPTER III

Phospholipase D Elevates the Level of MDM2 and Suppresses DNA Damage-Induced Increases in p53

INTRODUCTION

Cell proliferation is perhaps the most carefully regulated cellular activity. Protection from undesired proliferation prevents cancer and other proliferative disorders. The mechanisms through which cells overcome these protections have been aggressively investigated because they are frequently dysregulated in human cancer (Hanahan *et al* 2000). Many of the protections against cell proliferation involve cell cycle checkpoints where several criteria have to be met in order for a cell to continue through the cycle and divide (Iliakis *et al* 2003). It is at these checkpoints that decisions are made as to whether the cell should stop, proceed, or, when appropriate, undergo apoptosis. Mitogenic signaling involves the generation of signals that allow passage through cell cycle checkpoints. Since the default pathway for inappropriate cell proliferation signals is frequently apoptosis, some of the signals generated by mitogens have been termed "survival signals," since they prevent apoptosis.

The most studied survival signaling pathway involves the activation of phosphatidylinositol (PI) 3-kinase (PI3K), which generates PI-3,4,5-tris phosphate (PIP3). PIP3 production results in the recruitment and activation of Akt, a kinase that phosphorylates several key proteins that regulate apoptosis (Vivanco *et al* 2002). Recently, another phospholipid-modifying enzyme has been implicated in the generation of survival signals. This enzyme is phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline (Exton *et al* 2000). There are two PLD isoforms, PLD1 and PLD2 (Colley *et al* 1997 and Hammond, *et al* 1995), and both have been implicated in mitogenic signaling (Foster *et al* 2003).

PLD activity is elevated in response to platelet-derived growth factor (Plevin *et al* 1991), fibroblast growth factor (Motoike *et al* 1993), epidermal growth factor (EGF) (Song *et al* 1994), insulin (Karnam *et al* 1997), insulin-like growth factor 1 (Banno *et al* 2003), growth hormone (Zhu *et al* 2002), and sphingosine 1-phosphate (Banno *et al* 2001). PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src (Song *et al* 1991), v-Ras (Jiang *et al* 1995), v-Fps (Jiang *et al* 1994), and v-Raf (Frankel *et al* 1999). PLD activity was also able to induce a transformed phenotype in cells with elevated expression of a tyrosine kinase (Joseph 2001 and Lu *et al* 2000). Both PLD1 and PLD2 have been reported to induce anchorage-independent growth and enhance cell cycle progression of mouse fibroblasts (Ann 2003 and Min *et al* 2001). PLD activity prevented cell cycle arrest and apoptosis in cells with overexpressed Raf (Joseph *et al* 2002). PLD also prevented apoptosis in cells transformed by v-Src and in the MDA-MB-231 human breast cancer cell line (Zhong *et al* 2003). PLD activity also overcame apoptosis induced by H_2O_2 (Nozawa *et al* 2002) and glutamate (Kim *et al* 2003). Thus, an emerging role for PLD in the control of cell proliferation is to provide a survival signal that allows cells to avoid apoptosis under conditions of stress or inappropriate mitogenic signals (Foster *et al* 2003).

It was previously reported that PLD1 cooperated with c-Src to transform 3Y1 rat fibroblasts (Lu *et al* 2000). PLD1 also protected 3Y1 cells overexpressing c-Src from apoptosis (Zhong 2003), indicating that PLD1 generates a survival signal in these cells. The ability of PLD1 to cooperate with a tyrosine kinase such as c-Src is similar to the ability of simian virus 40 T antigen to cooperate with Ras to transform human cells (Hahn *et al* 1999). T antigen contributes to transformation by down-regulating the cell cycle

checkpoint proteins Rb and p53 (Pipas *et al* 2001). Thus, it is possible that PLD1 might similarly regulate control of cell cycle checkpoint proteins.

In this report, we describe studies of the effect of PLD activity upon p53 stabilization. We have taken advantage of the ability of 3Y1 cells overexpressing c-Src to tolerate elevated expression of PLD1 to demonstrate that PLD elevates basal MDM2 levels and suppresses DNA damage-induced increases in the stabilization of p53. The data provide evidence that the survival signals generated by PLD are mediated, at least in part, by suppression of p53 stabilization.

RESULTS

Elevated expression of PLD1 suppresses DNA damage-induced apoptosis.

It was reported previously that elevated PLD activity suppressed apoptosis induced by the withdrawal of serum in 3Y1 cells with elevated expression of c-Src (Zhong *et al* 2003). We wished to extend these studies to the apoptosis induced by DNA damage. As described above, elevated PLD1 expression is not tolerated well and actually stimulates apoptosis in normal rat fibroblasts deprived of serum (Zhong *et al* 2002). However, in the $3Y1^{c\text{-}Src}$ cells, PLD1 was not only tolerated, it caused transformation (Lu 2000 and Joseph *et al* 2002). Therefore, to alleviate problems associated with high levels of PLD1 expression, we developed a $3Y1^{c\text{-}Src}$-P1 cell line (Joseph *et al* 2002), this PLD inducible cell line stably overexpress c-Src, the expression and activity of PLD is induced by an inducer- Ponesterone A(PonA). These cells express elevated but tolerated levels of PLD1 in the absence of induction, and upon treatment with PonA, expression of PLD1 and PLD activity are both elevated by 16 h after treatment with PonA (Fig. 1). These data are consistent with the previously reported levels of PLD1 protein and activity levels in these cells (Joseph *et al* 2002). Having established that the $3Y1^{c\text{-}Src}$-P1 cells express elevated PLD1 and that expression could be further elevated by treatment with PonA, we subjected these and the parental $3Y1^{c\text{-}Src}$ cells to the DNA-damaging agent CPT and examined cell viability. As shown in Fig. 2A, the $3Y1^{c\text{-}Src}$-P1 cells were substantially more resistant to CPT than the parental $3Y1^{c\text{-}Src}$ cells. To investigate whether the loss of cell viability was due to apoptosis, we examined PARP cleavage in response to DNA damage, and as with cell viability, elevated PLD1 expression suppressed CPT-induced

PARP cleavage (Fig. 2B). These data suggest that elevated expression of PLD1 suppresses the apoptotic response to DNA damage.

Fig1

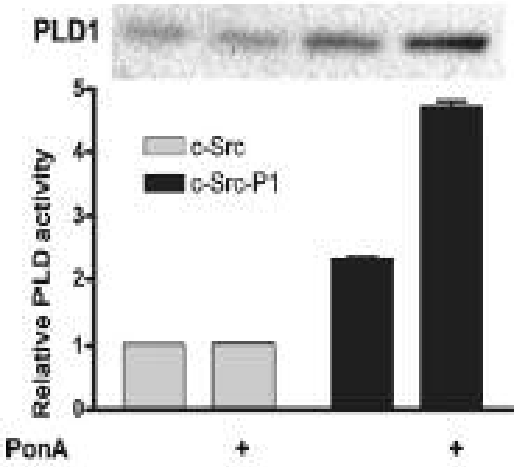

PLD1
Relative PLD activity
c-Src
c-Src-P1
PonA
+
+

Fig2

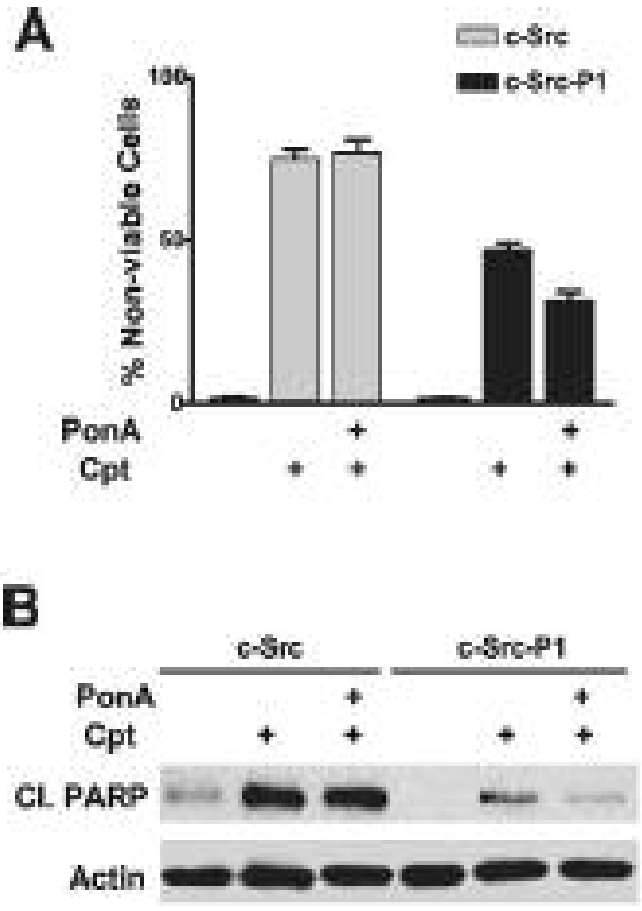

A
% Non-viable Cells
c-Src
c-Src-P1
PonA
Cpt
B
c-Src
c-Src-P1
PonA
Cpt
CL PARP
Actin

Fig1. Conditional expression of PLD1 in 3Y1$^{c\text{-}Src}$ cells. The construction of 3Y1$^{c\text{-}Src}$-P1 cells was described previously (Joseph *et al* 2002). PLD1 protein levels in the 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were determined in the presence and absence of PonA (10 μM, 20 h), as indicated, by Western blot analysis with an anti-PLD1 antibody (upper panel). Aliquots from these cells were also analyzed for PLD activity as described in Materials and Methods. The PLD activity values were normalized to untreated 3Y1$^{c\text{-}Src}$ cells. Error bars represent the standard deviation for triplicate samples from a representative experiment that was repeated two times.

Fig2. Elevated expression of PLD1 suppresses DNA damage-induced apoptosis. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were pretreated with PonA for 16 h. CPT was then added where indicated, and cell viability was examined 20 h later. The percentage of nonviable cells was determined by the uptake of trypan blue. Error bars represent the standard deviation for the average of data from three independent experiments. (B) Cell lysates from 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells prepared as described above were examined for the proteolytically cleaved PARP fragment (Cl. PARP) by Western blot analysis with an anti-PARP antibody. The data shown are representative of results obtained at least three times.

Elevated expression of PLD suppresses DNA damage-induced increases in the level of p53.

DNA damage stimulates the stabilization of p53, which in turn results in either cell cycle arrest or, if the damage is too extreme, apoptosis (Fei *et al* 2003). We therefore examined p53 levels induced by either CPT (Fig. 3A) or ADR (Fig. 3B) in the $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells in the presence and absence of PonA. The ability of the DNA-damaging agents to induce increased levels of p53 was substantially reduced in the $3Y1^{c\text{-}Src}$-P1 cells, and the induction of p53 was reduced even further when the cells were pretreated with PonA to increase PLD1 expression. These data indicate that elevated PLD activity in the $3Y1^{c\text{-}Src}$ cells, where PLD1 has been shown to provide a survival signal (Zhong *et al* 2003), suppresses DNA damage-induced increases in the level of p53.

While elevated expression of either PLD1 or PLD2 transforms 3Y1 cells with an overexpressed tyrosine kinase (Lu 2000 and Joseph *et al* 2001), elevated expression of either PLD1 or PLD2 in the parental 3Y1 cells that did not have elevated expression of c-Src induced apoptosis (Zhong *et al* 2002). We therefore examined the effect of elevated PLD1 expression on DNA damage-induced increases in p53 in the parental 3Y1 cells. The PonA-inducible expression system for PLD1 in 3Y1 cells (3Y1-P1 cells) was characterized previously (Zhong *et al* 2002). These cells were treated with either CPT or ADR in the presence and absence of PonA, as shown in Fig. 3A. In contrast with the $3Y1^{c\text{-}Src}$ cells, elevated PLD1 expression in the 3Y1 cells did not suppress the induction of p53 by either CPT or ADR (Fig. 3B). Thus, the ability of PLD1 to suppress the induction of p53 was apparently restricted to cells where elevated PLD activity was providing a survival signal.

It has recently been found that elevated PLD activity can provide a survival signal in MCF-7 human breast cancer cells (Chen 2003). We therefore examined the effect of CPT and ADR on p53 levels in MCF-7 cells and in MCF-7 cells that stably express PLD2. PLD2 was used in these cells rather than PLD1 because, as described previously (Chen 2003), PLD2 expression is tolerated by the MCF-7 cells better than PLD1. As shown in Fig. 3C, the ability of both CPT and ADR to increase the level of p53 was substantially reduced in MCF-7 cells that stably express PLD2 relative to the parental MCF-7 cells. The levels of PLD activity and PLD2 protein in the MCF-7 cells expressing PLD2 and MCF-7 cells transfected with the parental expression vector are shown in Fig. 3D. These data indicate that the effects of PLD upon p53 induction are not restricted to the 3Y1 cells with elevated expression of c-Src. The data also indicate that the ability of PLD to suppress induction of p53 may be restricted to cellular contexts where PLD is able to provide a survival signal.

We next examined whether the effect of PLD upon DNA damage-induced p53 was also reflected at the level of p53-targeted gene expression. A well-established transcriptional target of p53 is the cyclin kinase inhibitor p21 (Fei *et al* 2003). As shown in Fig. 4, DNA damage-induced increases in p21 were similarly suppressed in the $3Y1^{c\text{-}Src}$-P1 cells and further suppressed by the addition of PonA. These data further support the observation that elevated PLD activity suppresses DNA damage-induced increases in p53 levels and also argue that the p53 in these cells is wild type and capable of activating downstream p53 transcriptional targets (Fig. 3A).

Fig3

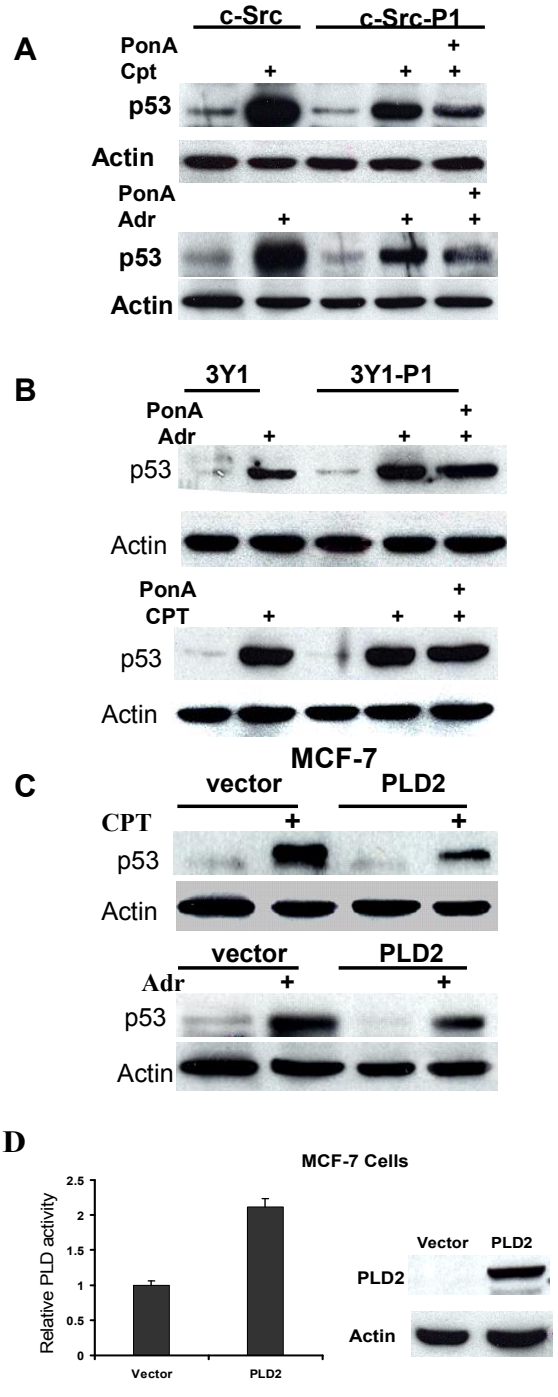

A
c-Src
c-Src-P1
PonA
Cpt
p53
Actin
PonA
Adr
p53
Actin
B
3Y1
3Y1-P1
PonA
Adr
p53
Actin
PonA
CPT
p53
Actin
C
MCF-7
vector
PLD2
CPT
p53
Actin
vector
PLD2
Adr
p53
Actin
D
MCF-7 Cells
Relative PLD activity
2.5
2
1.5
1
0.5
0
Vector
PLD2
Vector
PLD2
PLD2
Actin

Fig3. Elevated expression of PLD1 suppresses DNA damage-induced increases in the level of p53 in cells where PLD provides survival signals. (A) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells grown in DMEM with 10% bovine calf serum were treated with 5 μM CPT or 0.3 μM ADR for 4 h. The cells were then collected, and lysates were analyzed for p53 protein levels by using Western blot analysis with an anti-p53 antibody. Where indicated, PonA (10 μM) was added for 16 h prior tothe addition of CPT and ADR to increase the expression of PLD1 as described previously (Joseph *et al* 2001). (B) 3Y1 and 3Y1-P1 cells were treated with CPT, ADR, and PonA, and p53 levels were determined as described above. (C) MCF-7 cells stably transfected with pcDNA3.1(–)-PLD2 or the parental pcDNA3.1(–) vector were treated with either CPT or ADR, and p53 levels were determined as described above. (D) The relative levels of PLD2 protein determined by Western blot and PLD activity in the MCF-7 cells stably expressing PLD2 and transfected with the parental empty vector are shown. The data shown are representative of results obtained at least three times.

Fig4

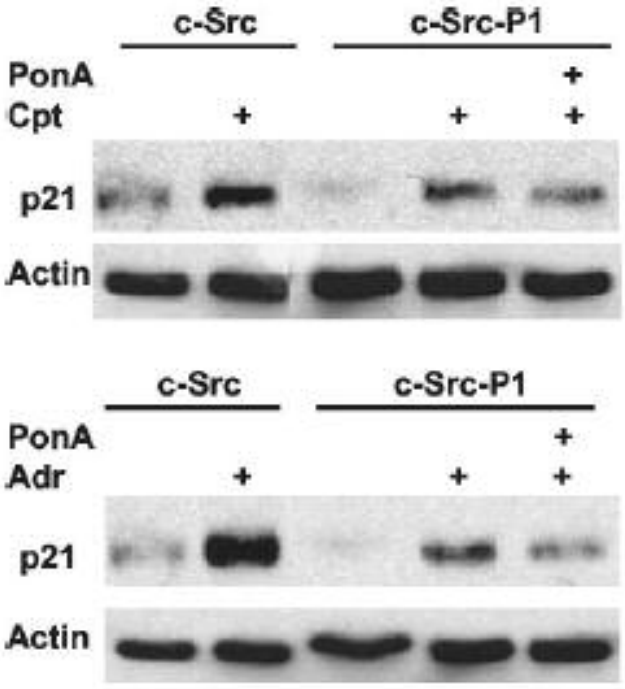
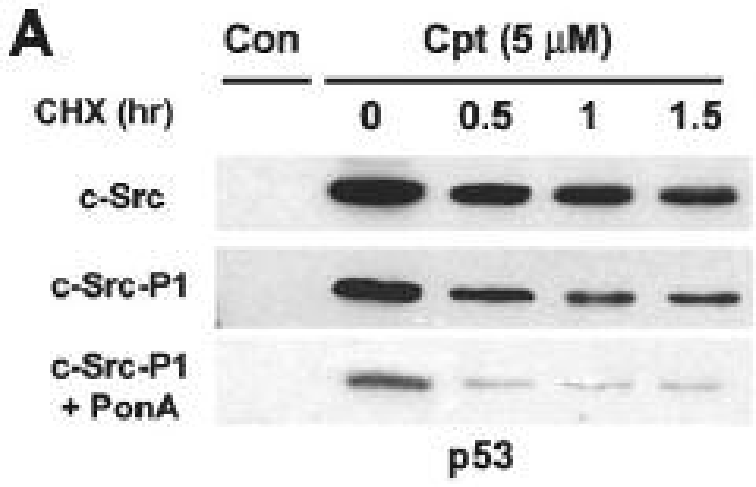
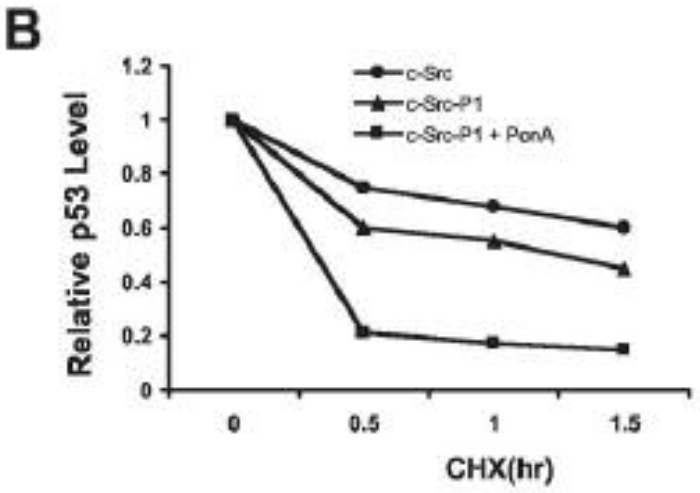


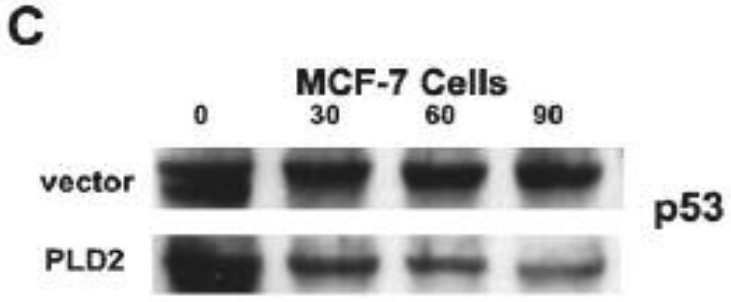
Fig4. Elevated expression of PLD1 suppresses DNA damage-induced increases of the level of p21. 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells grown in DMEM with 10% bovine calf serum were treated with 5 μM CPT or 0.3 μM ADR for 4 h. The cells were then collected, and lysates were analyzed for p21 protein levels by using Western blot analysis with an anti-p21 antibody. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CPT and ADR to increase the expression of PLD1 as described previously (Joseph *et al* 2001). The data shown are representative of results obtained twice.

Elevated expression of PLD1 accelerates degradation of p53. To investigate the mechanism of PLD1 inhibition of p53 induction, we first examined whether there was a difference in the half-life of p53 when PLD1 expression was elevated. $3Y1^{c\text{-}Src}$, $3Y1^{c\text{-}Src}$-P1, and PonA-treated $3Y1^{c\text{-}Src}$-P1 cells were subjected to CPT treatment (5 μM for 1 h). CHX (80 μg/ml) was then added to inhibit new p53 synthesis, and p53 levels were examined at 30-min intervals for 1.5 h (Fig. 3). As shown in Fig. 5A, the relative increases of the level of p53 induced by CPT were similar to those shown in Fig. 3. The relative p53 levels normalized to the level of p53 in the cells not treated with CHX (zero time point) was determined by using densitometer quantification of the data (Fig. 5A). As shown in Fig. 5B, p53 levels dropped substantially faster in the PonA-treated $3Y1^{c\text{-}Src}$-P1 cells. Similar data were obtained for the MCF-7 cells where the p53 turned over substantially faster in the MCF-7 cells overexpressing PLD2 (Fig. 5C and D). These data indicate that p53 is being degraded more rapidly in cells with elevated PLD activity and suggest that elevated PLD activity suppresses p53 expression, at least in part, by increasing the turnover of p53 protein.

Fig5


A
Con
Cpt (5 μM)
CHX (hr)
0
0.5
1
1.5
c-Src
c-Src-P1
c-Src-P1
+ PonA
p53


B
Relative p53 Level
1.2
1
0.8
0.6
0.4
0.2
0
c-Src
c-Src-P1
c-Src-P1 + PonA
0
0.5
1
1.5
CHX(hr)


C
MCF-7 Cells
0
30
60
90
vector
PLD2
p53

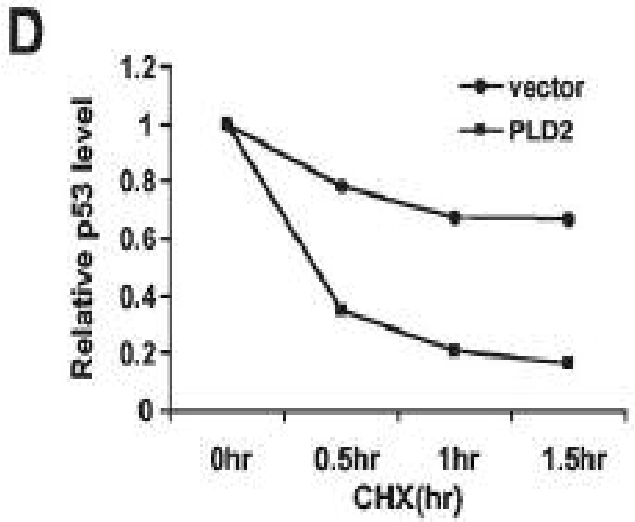


Fig5. Elevated PLD activity accelerates degradation of p53. (A) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells were treated with 5 μM CPT where indicated for 1 h. At this time, CHX (80 μg/ml) was added, and the cells were harvested at the indicated times. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CPT. Lysates were analyzed by Western blotting with anti-p53 antibody and, as a control, anti-tubulin antibody. (B) The results from the p53 data were analyzed by densitometer analysis, and the p53 levels normalized to the CPT-induced levels in the absence of CHX (zero time point) were determined. (C) MCF-7 cells and MCF-7 cells stably expressing PLD2 were treated with CHX as described above, and the levels of p53 were determined at the indicated times. (D) Densitometer analysis of the data in panel C is shown. The data shown are representative of results obtained three times.

Elevated expression of PLD1 increases basal MDM2 levels and inhibits DNA damage-induced decreases in MDM2.

The stability of p53 is regulated by the E3 ubiquitin ligase MDM2, which facilitates ubiquitination of p53 and targets it for degradation by the proteasome (30). We therefore examined the effect of elevated PLD1 expression upon MDM2 expression in the 3Y1$^{c\text{-}Src}$ cells. As shown in Fig. 6A, basal levels of MDM2 were elevated in the 3Y1$^{c\text{-}Src}$-P1 cells relative to those of the parental 3Y1$^{c\text{-}Src}$ cells. PonA treatment to further elevate PLD1 expression resulted in a further increase in the level of MDM2 in the 3Y1$^{c\text{-}Src}$-P1 cells. PonA had no effect upon MDM2 expression in the 3Y1$^{c\text{-}Src}$ cells (Fig. 6A). Both CPT and ADR treatment of the 3Y1$^{c\text{-}Src}$-P1 cells partially suppressed MDM2 expression (Fig. 6B). We also used transient transfection of PLD1 into the 3Y1$^{c\text{-}Src}$ cells, and as shown in Fig. 6A, this transfection led to increased MDM2 levels relative to a vector control. Both CPT and ADR suppressed MDM2 levels in the uninduced 3Y1$^{c\text{-}Src}$-P1 cells, and this suppression was reversed by the induction of PLD1 expression with PonA (Fig. 6B). Elevated expression of PLD2 in MCF-7 cells similarly led to increased basal human MDM2 (HDM2) expression (Fig. 6C). We also examined whether there was increased association between MDM2 and p53 in the 3Y1$^{c\text{-}Src}$-P1 cells, and as shown in Fig. 6D, increased expression of PLD1 resulted in increased levels of MDM2 in p53 immunoprecipitates. These data show that elevated expression of PLD leads to increased expression of the p53 E3 ubiquitin ligase MDM2 and increased association of MDM2 with p53. The data also explain, at least in part, the PLD-induced increase in p53 turnover as shown in Fig. 5.

Like p53, MDM2 is usually regulated at the level of stabilization (Michael *et al* 2003). We therefore compared the half-life of MDM2 in 3Y1$^{c\text{-}Src}$, 3Y1$^{c\text{-}Src}$-P1, and PonA-treated

3Y1$^{c\text{-}Src}$-P1 cells. The 3Y1$^{c\text{-}Src}$, 3Y1$^{c\text{-}Src}$-P1, and PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells were treated with CHX (80 μg/ml) to inhibit new MDM2 synthesis, and MDM2 levels were examined at 30-min intervals as in Fig. 5 (Fig. 7A).The relative MDM2 levels normalized to the level of MDM2 in the cells not treated with CHX (zero time point) were determined by using densitometer quantification of the data shown in Fig. 7A. As shown in Fig. 7B, MDM2 levels dropped substantially faster in the 3Y1$^{c\text{-}Src}$ cells than did those of the 3Y1$^{c\text{-}Src}$-P1 or PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells. These data indicate MDM2 is being degraded more slowly in cells with elevated PLD activity and suggest that PLD increases MDM2 expression, at least in part, by increasing the half-life of MDM2 protein.

Fig6

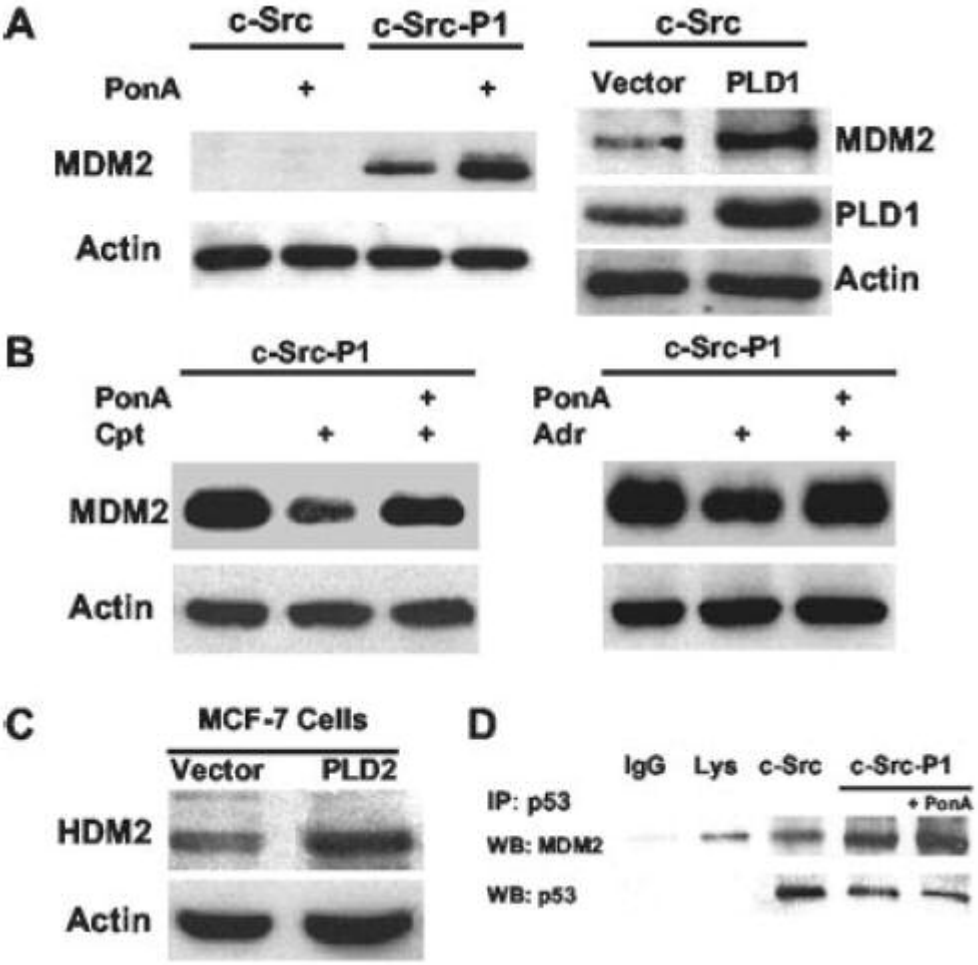


Fig6. Elevated expression of PLD1 increases basal MDM2 levels and inhibits DNA damage-induced decreases in MDM2. (A) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells were pretreated with PonA (10 μM) for 16 h where indicated, and the cells were then lysed and analyzed by Western blot analysis with an anti-MDM2 antibody. In the right panel, $3Y1^{c\text{-}Src}$ cells were transiently transfected with pCGN-PLD1 and the parental pCGN vector as indicated. MDM2 and PLD1 protein levels were determined by Western blot analysis. (B) $3Y1^{c\text{-}Src}$-P1 cells were pretreated with PonA where indicated, as described above. CPT (5 μM) or ADR (0.3 μM) was then added for 4 h where indicated, and lysates were examined for MDM2 expression as described above. (C) HDM2 expression levels were determined in MCF-7 cells and MCF-7 cells stably expressing PLD2 as described above. (D) Lysates from $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells were immunoprecipitated (IP) by using a mouse monoclonal p53 antibody. The p53 immunoprecipitates were then subjected to Western blot (WB) analysis with antibodies raised against HDM2 and p53 as indicated. A nonimmune immunoglobulin control immunoprecipitate (IgG) is shown, as is a portion of whole-cell lysate (Lys) that was not subjected to immunoprecipitation. The amount run on the gel was 4% of that used in the immunoprecipitates. PonA, where indicated, was included as described above. The data shown are representative of results obtained at least three times.

Fig7

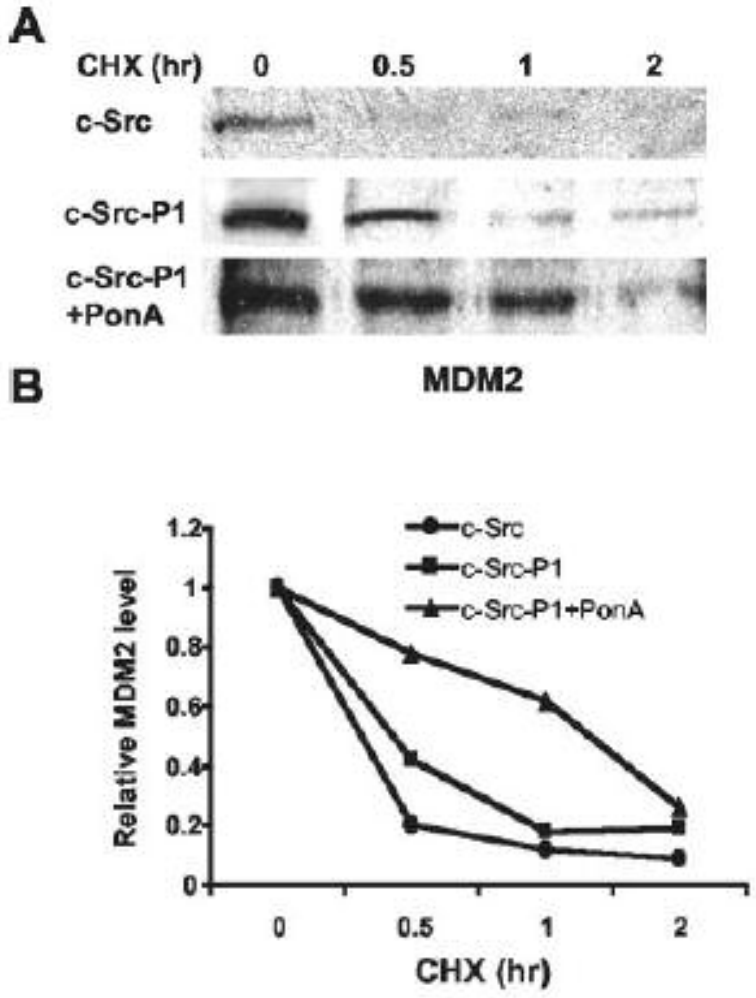


Fig7. Elevated expression of PLD1 increases the half-life of MDM2. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were treated with CHX (80 μg/ml), and the cells were harvested at the indicated times. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CHX. Lysates were analyzed by Western blotting with anti-MDM2 antibody and, as a control, antitubulin antibody. (B) The levels of MDM2 were determined by densitometer analysis and normalized to the level of MDM2 in the absence of CHX (zero time point). The data shown are representative of results obtained twice.

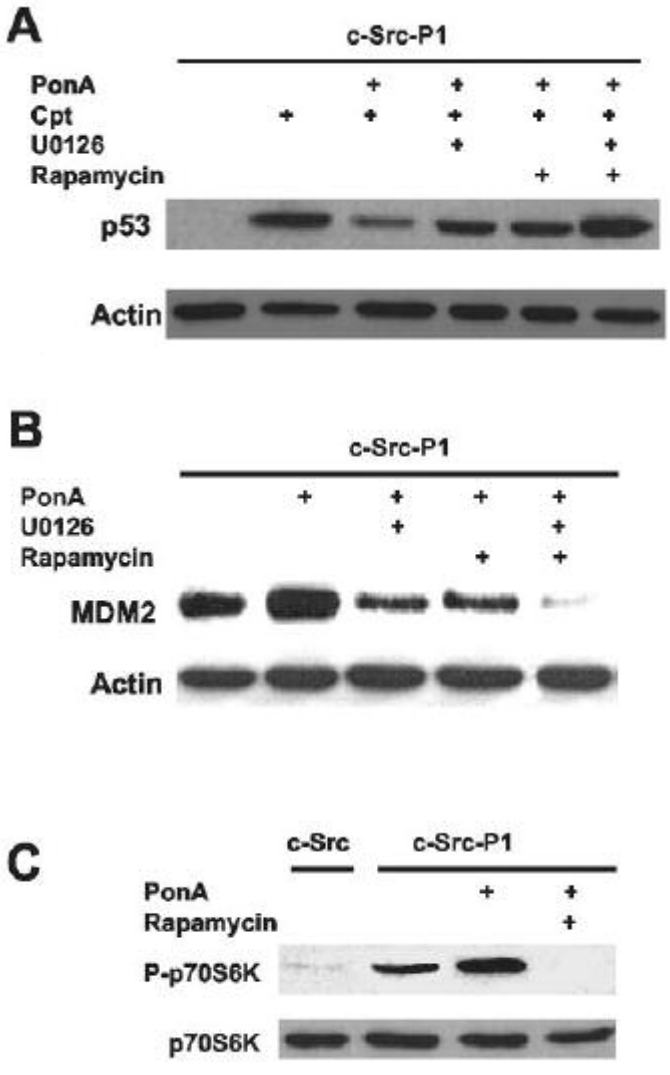
PLD1-induced increases in MDM2, suppression of p53, and apoptosis are dependent upon MAP kinase and mTOR.

It was previously reported that the activation of MAP kinase by EGF was dependent upon PLD (Shen *et al* 2001). mTOR (mammalian target of rapamycin) has also been reported to be dependent upon PLD-generated PA (Chen 2002, Fang 2001 and Chen 2003). We therefore examined the effect of inhibitors of MEK, the kinase that phosphorylates MAP kinase (U0126), and mTOR (rapamycin) on the PLD-stimulated effects on p53 stabilization. Surprisingly, we found that both U0126 and rapamycin inhibited the PLD-suppressed induction of p53 by CPT (Fig. 8A). When U0126 and rapamycin were added to the induced $3Y1^{c\text{-}Src}$-P1 cells together, the CPT-induced increase in p53 was restored almost to the level seen in the parental c-Src cells. Similarly, both U0126 and rapamycin inhibited the PLD-induced increases in MDM2, and treatment with U0126 and rapamycin together resulted in an even stronger reduction in MDM2 (Fig. 8B). We also found that another MEK inhibitor (PD98059) also prevented the effect of PLD upon p53 and MDM2 expression (data not shown). Fig 8C and 8D show that elevated PLD1 expression stimulated increases in the phosphorylation of mTOR substrate p70S6 kinase and MAP kinase and that these increases were sensitive to rapamycin and U0126, respectively. These data suggest that the PLD-induced effects upon PLD expression are dependent upon both mTOR and MAP kinase.

We next examined the effect of the MEK and mTOR inhibitors upon the suppression of DNA damage-induced apoptosis. The $3Y1^{c\text{-}Src}$ cells were subjected to CPT and PonA, and cell viability and PARP cleavage were determined (Fig. 2). The experiment was extended to investigate the effects of U0126 and rapamycin, and as shown in Fig. 9, cell viability was reduced with both U0126 and rapamycin. The effect of both U0126 and rapamycin

together was slightly greater than that with U0126 alone. When we examined PARP cleavage, the results resembled the effects of the drugs on MDM2 and p53 levels. Both U0126 and rapamycin substantially reversed the protective effect of PLD1 on PARP cleavage, and the two drugs together led to an apparent synergistic suppression of the inhibitory effect of PLD1 on PARP cleavage (Fig. 9). These data further support a role for both MAP kinase and mTOR in the PLD1 suppression of the p53 response pathway.

Fig8



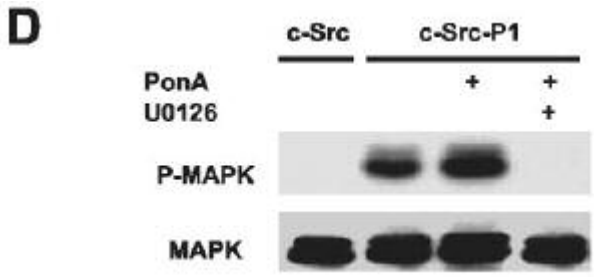


Fig8. PLD1-induced suppression of p53 and increases in MDM2 are reversed by inhibitors of MAP kinase and mTOR. (A) $3Y1^{c\text{-}Src}$-P1 cells were treated with CPT and PonA (Fig. 2A). Where indicated, U0126 (20 μM) and rapamycin (300 nM) were added 2 h prior to PonA treatment. Cells were then lysed and analyzed for the levels of p53 and actin by using Western blot analysis. (B) $3Y1^{c\text{-}Src}$-P1 cells were treated with PonA as described above. Where indicated, U0126 (20 μM) and rapamycin (300 nM) were added 2 h prior to PonA treatment. Cells were then lysed and analyzed for the levels of MDM2 and actin by using Western blot analysis. (C) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells were treated with PonA and rapamycin as indicated, as described above. Cell lysates were prepared, and the levels of S6 kinase (p70S6K) and phosphorylated S6 kinase (P-p70S6K) were determined by Western blot analysis with antibodies raised against p70S6K and p70S6K phosphorylated at Thr389. (D) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells were treated with PonA and U0126 as described above. Cell lysates were prepared, and the levels of MAP kinase (MAPK) and phosphorylated MAPK (P-MAPK) were determined by Western blot analysis with antibodies raised against MAPK and MAPK phosphorylated at Thr202/Tyr204. All of the data shown are representative of results obtained at least three times.

Fig9

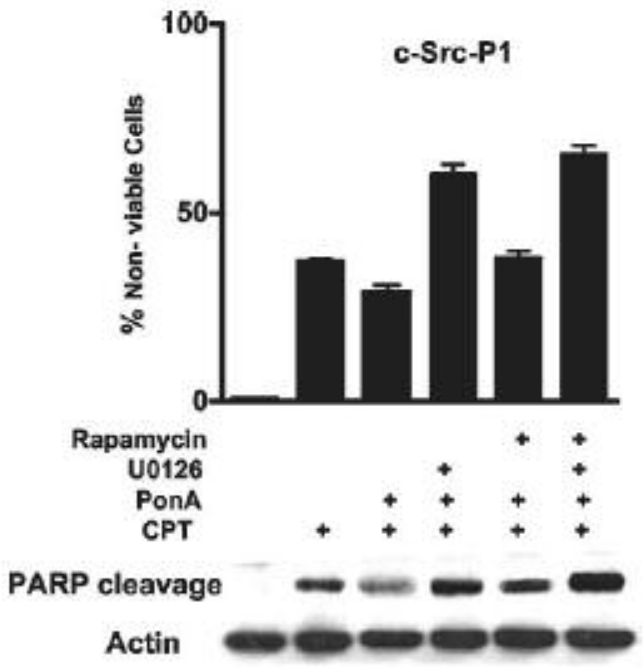


Fig9. PLD1 suppression of DNA damage-induced apoptosis is reversed by inhibitors of MAP kinase and mTOR. (A) 3Y1$^{c\text{-}Src}$-P1 cells were pretreated as indicated with PonA (16 h), and CPT was then added where indicated. U0126 (20 μM) and rapamycin (300 nM) were added 2 h prior to PonA treatment where indicated. Cell viability was then examined 20 h later. The percentage of nonviable cells and PARP cleavage were determined (Fig. 2). Error bars represent the standard deviation for the average of data from three independent experiments. The PARP cleavage data are representative of results obtained three times.

Elevated PLD activity does not stimulate the PI3K/Akt survival pathway, but basal PI3K activity is required for maximum stimulation of MDM2.

The above data indicate that elevated PLD activity stimulates mTOR activity, as indicated by the increased phosphorylation of S6 kinase. It has been shown by many that survival signals generated by the PI3K/Akt pathway also go through mTOR (Vivanco *et al* 2002). We therefore examined the effect of PLD activity upon Akt phosphorylation. As shown in Fig. 10A, elevated PLD1 expression had no effect on the phosphorylation state of Akt. However, the inhibition of basal Akt phosphorylation with the PI3K inhibitor LY294002 partially inhibited the PLD-stimulated increases in MDM2 (Fig. 10B). LY294002 completely inhibited both Akt and p70S6 kinase phosphorylation while having no inhibitory effect upon MAP kinase phosphorylation (Fig. 10B). These data indicate that while PLD1 does not stimulate PI3K activity and Akt phosphorylation, basal levels of PI3K and Akt are still important for the ability of PLD to increase MDM2 levels.

Fig10

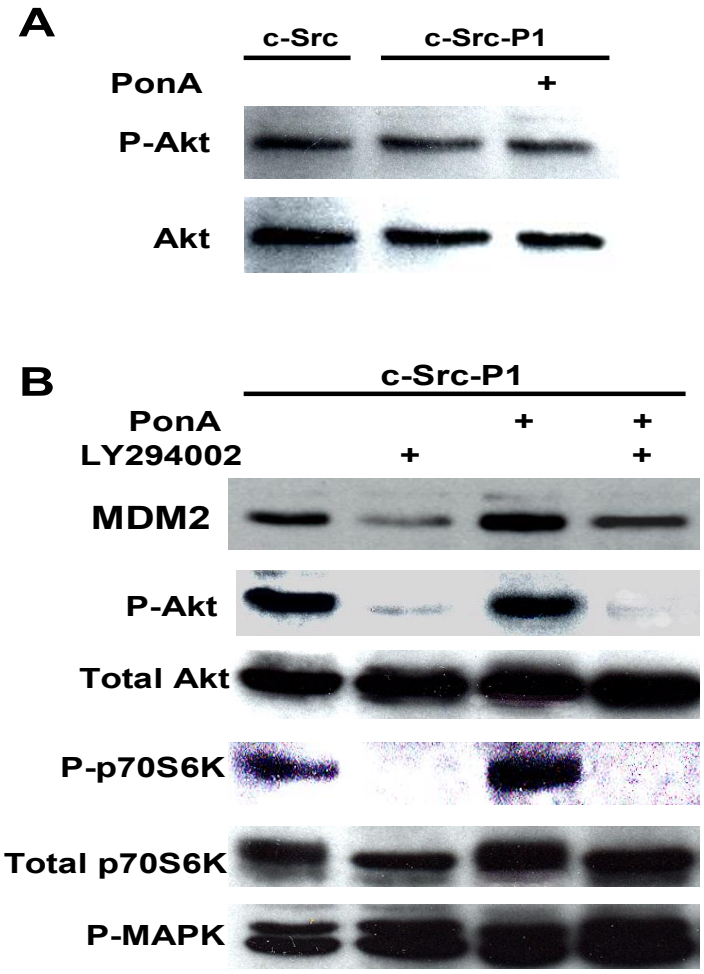


Fig10. Elevated PLD activity does not stimulate the PI3K/Akt survival pathway, but basal PI3K activity is required for maximum stimulation of MDM2. (A) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells were treated with PonA (Fig. 1). Cell lysates were prepared, and the levels of Akt and phosphorylated Akt (P-Akt) were determined by Western blot analysis

with antibodies raised against Akt and Akt phosphorylated at Ser473. (B) 3Y1$^{c\text{-}Src}$-P1 cells were treated with PonA and LY294002 (20 μM) as indicated. The LY294002 was added at the same time as the PonA 20 h prior to the preparation of cell lysates. The cell lysates were then subjected to Western blot analysis with the indicated antibodies (Fig. 7). The data shown are representative of results obtained at least two times.

DISCUSSION

These data have provided evidence that elevated PLD activity increased basal levels of MDM2 and suppressed p53 stabilization. This effect was restricted to cell contexts where PLD activity is able to provide a survival signal. The ability of PLD to suppress p53 stabilization was blocked by inhibitors of both MAP kinase and mTOR. Although PLD activity did not activate the PI3K/Akt pathway, inhibition of PI3K also inhibited PLD1-induced increases in MDM2 expression, indicating that basal PI3K/Akt activation contributes to the PLD-induced suppression of p53 stabilization. A model for the suppression of p53 stabilization through elevated basal expression of MDM2 is illustrated in Fig. 11. In this model, it is proposed that PLD1-induced increases in MAP kinase and mTOR synergistically elevate MDM2 levels and increase the turnover of p53. The activation of mTOR was also dependent upon basal levels of PI3K activity, suggesting a link between the PLD and PI3K survival pathways. Although much remains to be learned about the survival signaling pathways generated by PLD, the ability of PLD to suppress p53 stabilization provides a plausible mechanism for the reported survival signals generated by PLD (Joseph 2002, Foster 2003 and Zhong *et al* 2003).

The present study focused mostly on PLD1. PLD1 expression was reported to be elevated in breast cancer, and it was reported that there is elevated expression of PLD1 in the MDA-MB-231 cells, which have high levels of PLD activity (Zhong *et al* 2003). However, PLD2 has also been implicated in mitogenic and survival signals (Foster *et al* 2003). Moreover, PLD2 expression was reported to be elevated in renal cancer (Zhao *et al* 2000). As reported here, PLD2 also elevates MDM2 and suppresses DNA damage-

induced increases of p53 in MCF-7 cells. It has been hypothesized that PLD2 is activated in response to elevated PLD1 expression (Foster *et al* 2003). Consistent with this hypothesis, we have reported previously that both PLD1 and PLD2 can cooperate with elevated expression of a tyrosine kinase to transform cells (Lu 2000 and Joseph 2003). Thus, both PLD1 and PLD2 can apparently provide survival signals that suppress the activation of the p53 response.

A role for MAP kinase in the suppression of p53 stabilization is consistent with a previous report by Ries *et al*. (Ries *et al* 2000), who showed that oncogenic Ras suppressed p53 levels via MAP kinase. As shown here, elevated PLD activity led to increased phosphorylation of MAP kinase, and the PLD suppression of p53 stabilization was dependent upon MAP kinase. The activation of MAP kinase by PLD is likely to be indirect through the enhancement of receptor endocytosis. It was previously demonstrated that the activation of MAP kinase in response to EGF requires receptor endocytosis (Vieira 1996 and Kranenburg 1999), and endocytosis of the EGF receptor and MAP kinase activation were both dependent upon PLD activity (Shen 2001). Importantly, elevated PLD activity increased basal levels of receptor endocytosis in the absence of EGF (Shen *et al* 2001). The proposed complex stimulation of MAP kinase by PLD is also reflected in Fig. 11.

It was previously proposed that survival signals generated by either PLD or PI3K represented alternative survival pathways that were related by a dependence upon PI-4,5-bisphosphate, a cofactor for PLD and a substrate for PI3K (Foster *et al* 2003). The two pathways are also related in that they both target mTOR (Schumelze 2000 and Chen *et al* 2002). The evidence presented here, that elevated PLD activity does not elevate Akt

phosphorylation, is consistent with this hypothesis in that PLD activity does not activate the PI3K/Akt signaling pathway. However, interestingly, the elevation of MDM2 by PLD was partially inhibited by inhibiting basal PI3K activity, indicating a linkage between the PLD and PI3K survival pathways. PLD-induced suppression of p53 stabilization was sensitive to rapamycin, implicating mTOR as a target of PLD. Although PLD can apparently stimulate mTOR without increasing Akt phosphorylation (Fang 2001, Chen 2002 and Chen 2003), there appears to be a requirement for a basal level of Akt activity since the inhibition of PI3K led to a partial inhibition of the PLD-induced increases in the level of MDM2. Inhibition of PI3K completely inhibited the phosphorylation of p70S6 kinase. Thus, the effect of inhibiting PI3K is likely due to an Akt requirement for the activation of mTOR. These data further suggest a link between the PLD and PI3K/Akt survival pathways in that both appear to target mTOR. There appears to be an Akt requirement for PLD survival signals, and there is also likely a PLD requirement for PI3K/Akt signals through mTOR since mTOR has a requirement for PLD-generated PA (Fang 2001 and Chen 2002).

The ability of PLD to suppress p53 stabilization can explain, at least in part, the ability of PLD to suppress apoptosis (Joseph 2002 and Zhong 2003) and to cooperate with tyrosine kinases to transform cells (Lu 2000 and Joseph 2001). Tyrosine kinase activity is commonly elevated in a variety of human cancers, especially breast cancer, where there is elevated expression of tyrosine kinases such as the EGF receptor, Her2/Neu, and c-Src (Biscardi 1999). Interestingly, elevated expression of PLD1 has been reported to be common in breast cancer tissues (Uchida 1997 and Noh 2000). PLD activity has also been reported to be elevated in renal and gastric cancers (Uchida 1999 and Zhao 2000),

and a polymorphism of the PLD2 gene was recently reported to be associated with the prevalence of colorectal cancer (Yamada 2003). Those reports suggest that elevated PLD activity may be providing a survival signal in these cancers. The data provided here reveal that PLD can suppress the level of a tumor suppressor that has been implicated in as much as 50% of human cancers. Elevated levels of MDM2 have also been reported in a substantial number of human cancers (reviewed by Chene [Chene 2003]). Data presented here indicate that elevated PLD activity may be responsible for the presence of high levels of MDM2 in some of these cancers. The finding of elevated PLD activity in virtually all cancer types where it has been investigated combined with the observation that PLD is able to provide survival signals in cancerous or transformed cells (Zhong 2003), suggests that elevated PLD activity in cancer cells is important for their survival. The ability of PLD to increase MDM2 and suppress p53 stabilization provides strong support for this hypothesis.

Fig11

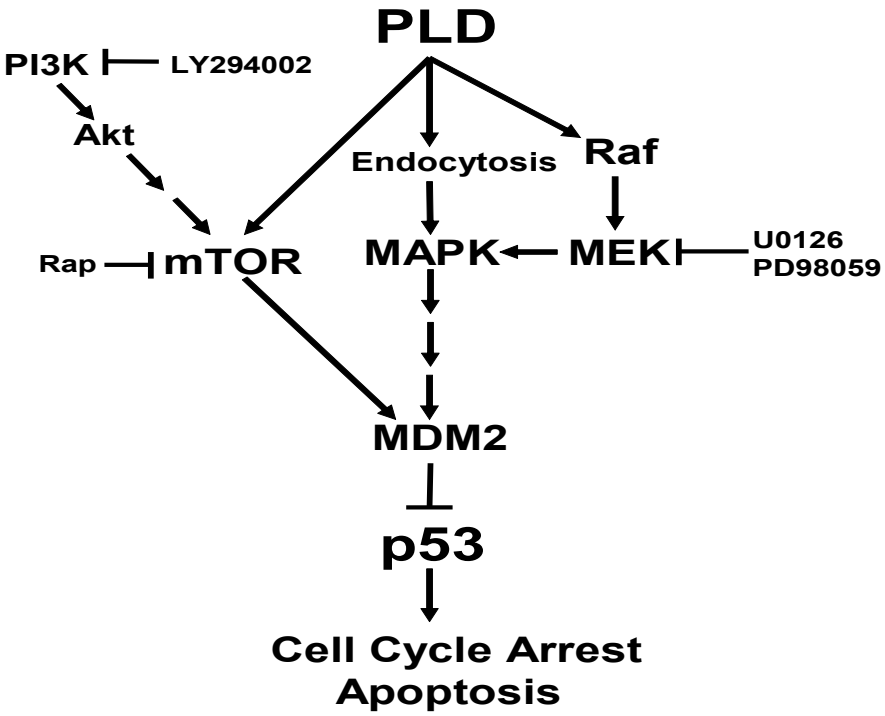


Fig11. Schematic model for suppression of p53 stabilization by PLD. It is proposed that the ability of PLD to suppress p53 expression is mediated by increasing the expression of the E3 ubiquitin ligase MDM2. The increased expression of MDM2 is dependent upon PLD1-induced increases in both mTOR and the Raf/MEK/MAP kinase cascade. Both mTOR (Fang 2001 and Chen 2003) and Raf (Ghosh 1996 and Rizzo 1999) have been reported to directly interact with and be affected by PA. PLD has also been reported to be required for endocytosis of the EGF receptor (Shen 2001), and endocytosis of activated MEK has been reported to be required for the activation of MAP kinase (Kranenburg 1999 and Shen 2001). Thus, it is proposed that PLD is required for the activation of both

MAP kinase and mTOR, which work synergistically to elevate MDM2 and suppress p53 stabilization. Basal levels of PI3K activity and Akt are also partially required for elevated expression of MDM2, although PLD activity does not lead to increased Akt phosphorylation.

CHAPTER IV

mTOR-dependent Suppression of Protein Phosphatase 2A is critical for Phospholipase D Survival Signals in Human Breast Cancer Cells

INTRODUCTION

Mitogenic signaling involves the generation of signals that allow passage through cell cycle checkpoints. Since the default pathway for inappropriate cell proliferation signals is frequently apoptosis, some mitogenic signals have been termed "survival signals" since they prevent default apoptotic programs (Downward *et al* 2004). Survival signals are frequently dysregulated in human cancer (Vivanco 2002, Luo *et al* 2003). Phospholipase D (PLD)[1], which is elevated in several human cancers (Foster *et al* 2003), generates a survival signal that suppresses apoptosis in human breast cancer cells Zhong 2003, Chen *et al* 2005). Interestingly, PLD targets mTOR, the mammalian target of rapamycin (Schmelzie *et al* 2000), which has a requirement for the PLD metabolite phosphatidic acid (Fang *et al* 2001). PLD stimulates increased mTOR activity and the phosphorylation of mTOR targets such as ribosomal subunit S6-kinase (Fang 2003, Hui 2004 and Chen *et al* 2005). Survival signals generated by PI3K also target mTOR indirectly through activation of Akt kinase (Cantly *et al* 2002). Thus, mTOR appears to be a common target of both PLD- and PI3K-generated survival signals.

The critical targets of mTOR for survival signals have not been fully established, although the translational machinery activated by mTOR has been suggested (Ruggero 2003, Avdulov *et al* 2004). mTOR phosphorylates S6-kinase and 4E-BP1 (Hay *et al* 2004) to enhance translation of select mRNA transcripts (Schmelzie *et al* 2000). Another target of mTOR implicated in the regulation of protein translation is PP2A (Janssens *et al* 2001). PP2A dephosphorylates the mTOR substrates S6-kinase and 4E-BP1 (Peterson *et al* 1999). Suppression of PP2A could be highly significant since the transformation of

human cells by the combination of H-Ras and SV40 early region genes (Hanh *et al* 1999) was shown to require the SV40 small t-antigen (Hahn 2002, Chen *et al* 2003), which interacts with and suppresses PP2A (Pallas 1990, Yang 1991 and Chen *et al* 2004). Based on these studies, it has been proposed that PP2A is a tumor suppressor gene (Van Hoof *et al* 2004). In this regard, it is of interest that PLD, like SV40 early region genes, cooperates with signaling oncogenes to transform rat fibroblasts in culture (Lu 2000, Joseph *et al* 2001). The transformation of human cells with SV40 early region genes also requires the large T-antigen, which interacts with and suppresses p53 (Hahn *et al* 1999), and in this regard it may be of significance that elevated PLD activity stimulates an mTOR-dependent increase in the expression of MDM2, which suppresses the induction of p53 (Hui *et al* 2004).

The ability of PLD to cooperate with a signaling oncogene to transform cells (Lu 2000, Joseph *et al* 2001) and to suppress p53 expression (Hui *et al* 2004) suggests that PLD is able to achieve much of what SV40 early region genes accomplish in cell transformation and tumorigenesis. We therefore asked whether elevated PLD activity, like SV40 small t-antigen, suppresses PP2A. We report here that the elevated PLD activity in the human breast cancer cell line MDA-MB-231 causes an mTOR-dependent suppression of PP2A that is critical for the survival signals generated by PLD.

RESULTS

PP2A activity in human breast cancer cells inversely correlates with the level of PLD activity

The human breast cancer cell lines MCF7 and MDA-MB-231 have been widely used as breast cancer cells with less aggressive and more malignant phenotypes respectively (Lacroix *et al* 2004). We reported previously that there is elevated PLD activity in the MDA-MB-231 cells relative to the MCF7 cells, and that this PLD activity provided a survival signal that suppressed apoptosis in cells deprived of serum (Zhong *et al* 2003). Since the suppression of PP2A has been implicated in the transformation of human cells (Foster 2004, Chen *et al* 2004), we examined the level of PP2A activity in the MCF7 and MDA-MB-231 cells. As shown in Fig. 1A, there was an inverse correlation between the level of PLD activity and PP2A activity in these cells. MDA-MB-231 cells, with about 8-fold higher PLD activity than the MCF7 cells, had less than half the level of PP2A activity observed in the MCF7 cells. PP2A is (reversibly) methylated throughout the cell cycle and is demethylated at the G1/S cell cycle boundary (Turowski *et al* 1995), where PLD has been speculated to promote cell cycle progression (Foster *et al* 2003). It has also been reported that methylated PP2A has increased phosphatase activity (Favre *et al* 1994). We therefore examined the methylation state of PP2A using an antibody that recognizes unmethylated PP2A. As shown in the lower panel of Fig. 1A, there was almost four fold more unmethylated PP2A in the MDA-MB-231 cells relative to the MCF7 cells, further indicating that PP2A is down regulated in the MDA-MB-231 cells. Thus, the level of both PP2A activity and PP2A methylation status correlates inversely with the level of PLD activity in the MDA-MB-231 and MCF-7 cells.The data in Fig. 1A

suggest the possibility that PLD activity in the MDA-MB-231 cells suppresses PP2A activity. To test this more directly, we examined the PLD and PP2A activity in MDA-MB-231 cells and in MDA-MB-231 cells stably expressing a catalytically-inactive dominant negative K758R mutant of PLD2 (231-P2DN) (Colley *et al* 1997), which we have used previously to suppress the survival signals in MDA-MB-231 cells (Zhong 2003, Chen *et al* 2003). As shown in Fig. 1B, the dominant negative PLD2 reduced PLD activity to about 20% that observed in the vector control MDA-MB-231 cells. The dominant negative PLD also increased the level of PP2A activity and decreased the level of unmethylated PP2A four fold (Fig. 1B). These data indicate that suppression of PP2A activity in the MDAMB-231 cells is dependent upon the elevated PLD activity in these cells.We next investigated whether increasing PLD activity in MCF7 cells would suppress PP2A activity. We generated MCF7 cells stably expressing elevated PLD2 (MCF7-P2 cells). As shown in Fig. 1C, cells with elevated PLD2 had about seven-fold higher levels of PLD activity than empty vector control MCF7 cells (MCF7-v). The elevated PLD activity also suppressed PP2A activity and increased the level of methylated PP2A (Fig. 1C).We previously demonstrated that both PLD1 and PLD2 can provide survival signals in MDA-MB-231 cells (Zhong 2003). We therefore also examined the effect of PLD1 on PP2A activity in MDA-MB-231 and MCF7 cells. Since stable expression of PLD1 and PLD1 mutants is generally not tolerated by cells as well as PLD2, we used transient, rather than stable expression of PLD1 and a catalytically-inactive dominant negative K898R mutant of PLD1. As shown in Fig1D, the dominant negative PLD1 mutant, like PLD2, suppressed PLD activity, elevated PP2A activity, and reduced the level of unmethylated PP2A in MDA-MB-231 cells. And as shown in Fig.

1E, transient expression of wild type PLD1 in MCF7 cells suppressed PP2A activity and increased the level of unmethylated PP2A. Thus, both PLD1 and PLD2 can suppress PP2A activity. Collectively, the data in Fig. 1 show a strong correlation between elevated PLD activity and the suppression of PP2A activity.

Fig 1

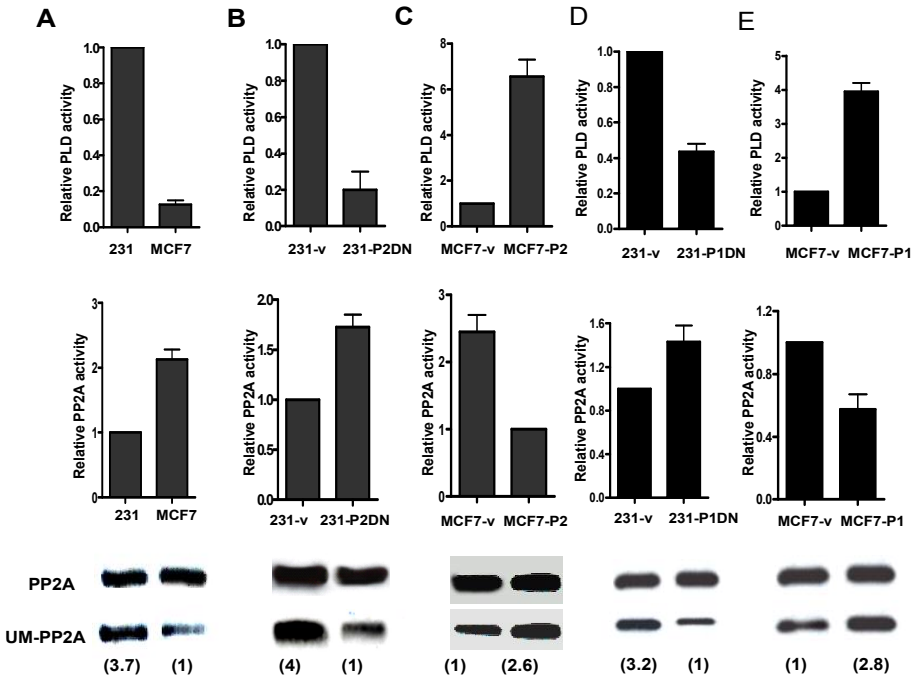


Fig1. PP2A activity in human breast cancer cells inversely correlates with the level of PLD activity**.** *A,* The levels of PLD (upper panel) and PP2A (middle panel) activity were determined as described in Experimental Procedures. The PLD and PP2A activity in the MCF7 cells relative to the PLD and PP2A activity in the MDA-MB-231 (231) cells was determined. Error bars represent the standard deviation for triplicate samples from a representative experiment repeated 3 times. In the lower panel, the level of unmethylated PP2A (UM-PP2A) and the level of total PP2A were determined by Western blot analysis

using antibodies that recognize either un-methylated (inactive) or total PP2A. The unmethylated PP2A band was determined by densitometer tracing and the relative level of unmethylated PP2A normalized to the total PP2A is shown in parentheses. *B,* The levels of PLD activity, PP2A activity, and un-methylated PP2A were determined as in *A* in MDA-MB-231 cells that were stably transfected with either an emptor vector (231-v) as a control or a vector expressing a catalytically inactive dominant negative mutant of PLD2 (P2DN). *C,* The levels of PLD activity, PP2A activity, and unmethylated PP2A were determined in MCF7 cells that were stably transfected with either an empty vector control (MCF7-v) or a vector expressing wild type PLD2 (MCF7-P2). *D,* MDA-MB-231 cells were transiently transfected with either an empty vector control or with a vector expressing dominant negative PLD1. PLD activity, PP2A activity and unmethylated PP2A levels were determined 48 h later as in 1B. *E,* MCF7 cells were transiently transfected with either an empty vector control or with a vector expressing wild type PLD1. PLD activity, PP2A activity and unmethylated PP2A levels were determined 48 h later as in 1C. All experiments shown are representative of ones repeated 3 times.

Suppression of PP2A by PLD is dependent upon mTOR

We previously reported that PLD2 could provide a rapamycin-sensitive survival signal in MCF7 cells implicating mTOR as a mediator of the PLD-generated survival signals (Chen *et al* 2005). Rapamycin has been reported to block the dephosphorylation of S6-kinase and to increase PP2A activity (Peterson *et al* 1999), possibly by preventing phosphorylation of a PP2A regulatory subunit α4 (mammalian homologue of yeast TAPPolunovsky *et al* 2000) (Janssens *et al* 2001). We therefore examined the effect of suppressing mTOR on the PLD-dependent suppression of PP2A activity in MDA-MB-231 and the MCF-P2 cells with elevated PLD2 expression. Two approaches were employed, the first being the effect of rapamycin on PP2A activity in the two cell types. As shown in Fig. 2A, rapamycin increased PP2A activity in both the MDA-MB-231 and MCF7-P2 cells. The lower panel of Fig. 2A shows that the rapamycin was working since phosphorylation of the mTOR substrate S6-kinase was blocked by rapamycin. As reported previously (Chen *et al* 2003), elevated PLD activity increases that amount of rapamycin required to suppress mTOR and MDA-MB-231 cells have very high levels of PLD activity that requires high concentrations of rapamycin to suppress mTOR (Chen *et al* 2003). To establish that the high concentration of rapamycin (20 μM) used here was due to an effect on mTOR and not another cellular target, we introduced rapamycin resistant mTOR mutant used previously to demonstrate the specificity of rapamycin (Chen *et al* 2003) into the MDA-MB-231 cells and examined the effect of rapamycin on PP2A activity. As shown in Fig. 2B, the rapamycin resistant mTOR mutant reversed the effect of the high concentration of rapamycin used indicating that the effect of rapamycin was on mTOR and not another cellular target. We also used mTOR siRNA to suppress

mTOR expression, and as shown in Fig. 2C, the mTOR siRNA reduced mTOR expression (lower panel) and increased PP2A activity in both the MDA-MB-231 and MCF7-P2 cells. These data indicate that suppression of PP2A by PLD is dependent upon mTOR.

Fig2

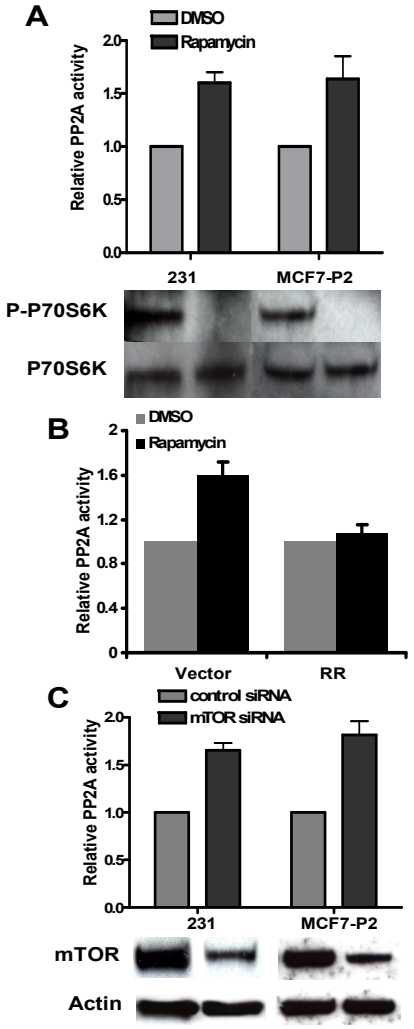


Fig2. Suppression of PP2A by PLD is dependent upon mTOR**.** *A,* MDA-MB-231 and MCF7-P2 cells were treated with rapamycin (20 μM for 1 hr at which time PP2A activity

was determined. The effect of rapamycin treatment on the phosphorylation state of the mTOR substrate S6-kinase was determined by Western blot using antibodies against phosphorylated S6-kinase (P- p70S6K) and S6-kinase (p70S6K). *B,* MDA-MB-231 cells transiently transfected with either an empty vector control or pcDNA3-mTOR-RR which expresses a rapamycin resistant mutant of mTOR (Brunn *et al* 1997), were treated with rapamycin (20 μM for 1 hr at which time PP2A activity was determined as in *A*. *C,* MDA-MB-231 and MCF-P2 cells were transfected with mTOR siRNA or a control siRNA and PP2A activity was determined 72 hr later. The effect of siRNA on the level of mTOR was determined by Western blot using an anti mTOR antibody. Error bars represent the standard deviation for triplicate samples from a representative experiment repeated 3 times. The experiments are representative of those repeated 3 times.

PLD suppresses the association between the PP2A catalytic subunit and both S6K and 4E-BP1.

The catalytic subunit of PP2A (PP2A-C) has been reported to associate with and dephosphorylate S6-kinase (Ballow *et al* 1988). PP2A-C has also been implicated in the dephosphorylation of 4E-BP1 (Peterson *et al* 1999). We therefore examined the association between the PP2A and S6-kinase and 4E-BP1 in MCF-v and MCF-P2 cells. Lysates from these cells were immunoprecipitated with antibodies raised against either S6-kinase or 4E-BP1, followed by Western blot analysis using an antibody raised against the PP2A catalytic subunit. As shown in Fig. 3A, high levels of PP2A could be detected in both the S6-kinase and 4E-BP1 immunoprecipitates from the MCF7 cells. In contrast, very little PP2A could be detected in the immunoprecipitates from the MCF7-P2 cells. Treating the MCF7-P2 cells with rapamycin restored the association between PP2A and

both S6-kinase and 4E-BP1 (Fig. 3A). We next examined the association between PP2A and both S6-kinase and 4E-BP1 in MDA-MB-231 cells and the MDA-MB-231 cells expressing the dominant negative PLD2 mutant. As shown in Fig. 3B, there was very little co-precipitation of PP2A with either S6-kinase or 4E-BP1 from MDA-MB-231 cell lysates. However, in the MDA-MB-231 cells expressing the dominant negative PLD2, association between PP2A and both S6-kinase and 4E-BP1 was restored (Fig. 3B). These data further suggest that PLD suppresses PP2A.

Fig3

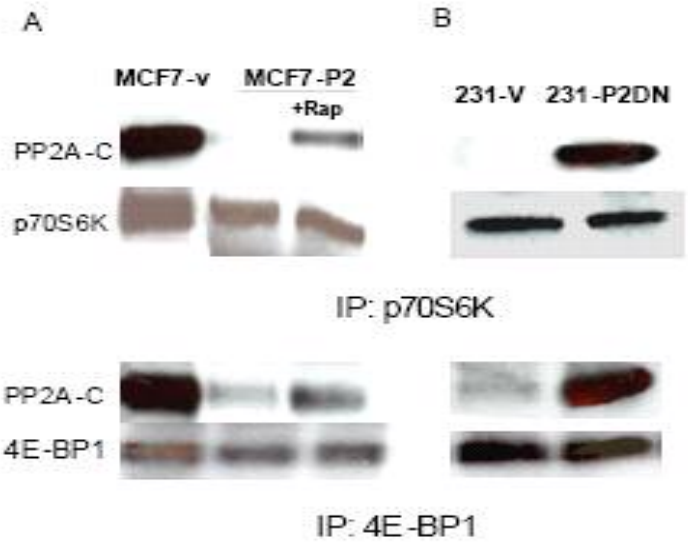


Fig3. PLD suppresses the association between PP2A catalytic subunit and both S6-kinase and 4E-BP1. *A,* Lysates from MCF7-v and MCF7-P2 cells were immunoprecipitated with antibodies to either S6-kinase (upper panel) or 4E-BP1 (lower panel). Immunoprecipitates were subjected to Western blot analysis using antibodies raised against PP2A-C and either S6-kinase (upper panel) or 4E-BP1 (lower

panel). Where indicated, rapamycin (20 μM) was added to the MCF-P2 cells 1 hr prior to preparation of cell lysates. *B,* Lysates from 231-v and 231-P2DN cells were to immunoprecipitated with either anti-S6-kinase antibody (upper panel) or with anti-4E-BP1 antibody (lower panel) and the immunoprecipitates were subjected to Western blot analysis using an antibodies raised against the catalytic subunit of PP2A and either S6-kinase (upper panel) or 4E-BP1 (lower panel) as in *A*.

SV40 small t-antigen or suppression of PP2A activity restores survival of MDA-MB-231 cells lost through inhibition either PLD or mTOR

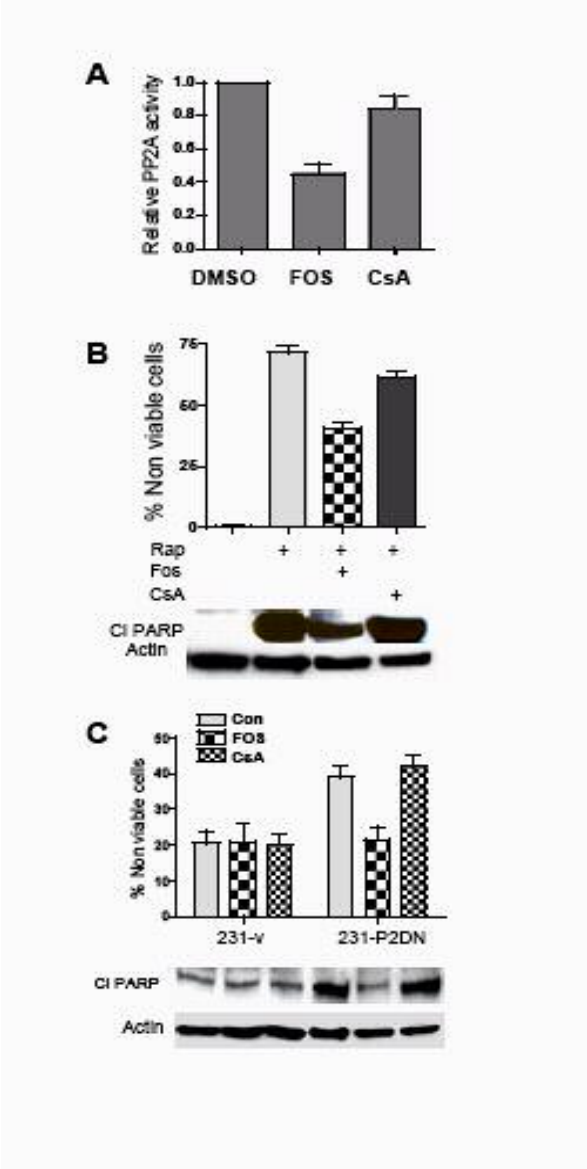
The data presented above indicate that PLD suppresses PP2A in an mTOR-dependent manor. We demonstrated previously that PLD suppresses apoptosis in MDA-MB-231 cells subjected to serum withdrawal (Zhong *et al* 2003) and this suppression is dependent on mTOR (Chen *et al* 2003). This raises the question as to whether the mTOR-dependent suppression of PP2A is required for the survival signals generated by PLD. To address this question, we examined the effect of the PP2A inhibitor fostriecin (FOS) on MDA-MB-231 cells where the survival signals generated by PLD have been blocked. We first examined the effect of FOS on PP2A activity in the MDA-MB-231 cells, and as shown in Fig. 4A, FOS suppressed PP2A activity, whereas the PP2B inhibitor cyclosporine A had no effect. We then examined the effect of FOS on the survival of MDA-MB-231 cells subjected to serum withdrawal in the presence of rapamycin. Rapamycin blocks PLD-generated survival signals and induces apoptosis in MDA-MB-231 cells subjected to serum withdrawal (Chen *et al* 2003). As shown in Fig. 4B, these cells were highly sensitive to rapamycin in the absence of serum as indicated by the loss of cell viability and increased cleavage of the caspase 3 substrate PARP. Thus, blocking the mTOR-

dependent survival signal generated by PLD in these cells in the absence of serum results in apoptosis. However, if FOS is present, the effect of rapamycin was substantially reduced (Fig. 4B). The PP2B inhibitor was not able to suppress apoptosis under these conditions. We also examined the effect of FOS on the survival of MDA-MB-231 cells expressing the dominant negative PLD2. As shown in Fig. 4C, these cells, with suppressed PLD activity, were highly sensitive to the withdrawal of serum as indicated by the loss of cell viability and increased PARP cleavage relative to vector control cells. Thus, expression of the dominant negative PLD2 blocks the survival signal generated by PLD in these cells and the removal of serum now results in apoptosis. However, if FOS was present, then the MDA-MB-231 cells expressing the dominant negative PLD2 cells survived as well as the vector control MDA-MB-231 cells when serum is withdrawn. The PP2B inhibitor did not suppress apoptosis under these conditions. These data provide evidence that the suppression of PP2A by elevated PLD activity in MDA-MB-231 cells is critical for the survival signals generated by PLD in these cells.

Transformation of human cells with SV40 early region genes required small t-antigen (Hahn *et al* 2002), which interacts with and suppresses PP2A (Van Hoof *et al* 2004). We therefore investigated whether SV40 small t-antigen could rescue survival of serum-starved MDA-MB-231 cells expressing the dominant negative PLD2. This experiment was performed in two ways: MDA-MB-231 cells were transiently transfected with a vector that expresses the dominant negative PLD2 (P2DN) or the dominant negative PLD2 in combination with vectors expressing SV40 small t-antigen or a small t-antigen mutant that does not interact with PP2A (Sontag 1993, Frost *et al* 1994). As shown in Fig. 5A, the dominant negative PLD2 increased cell death and PARP cleavage as in Fig.

4 and as described previously (Chen *et al* 2003). Co-transfection with the SV40 small t-antigen vector reduced cell death and PARP cleavage, whereas the vector expressing the SV40 small t mutant did not. A similar experiment was performed on MDA-MB-231 cells stably expressing dominant negative PLD2, and as shown in Fig. 5B, SV40 small t-antigen, but not the small t-antigen mutant reduced cell death and PARP cleavage in these cells. These data further indicate that PP2A is a critical target of PLD survival signals in MDA-MB-231 cells.

Fig4 **Fig5**



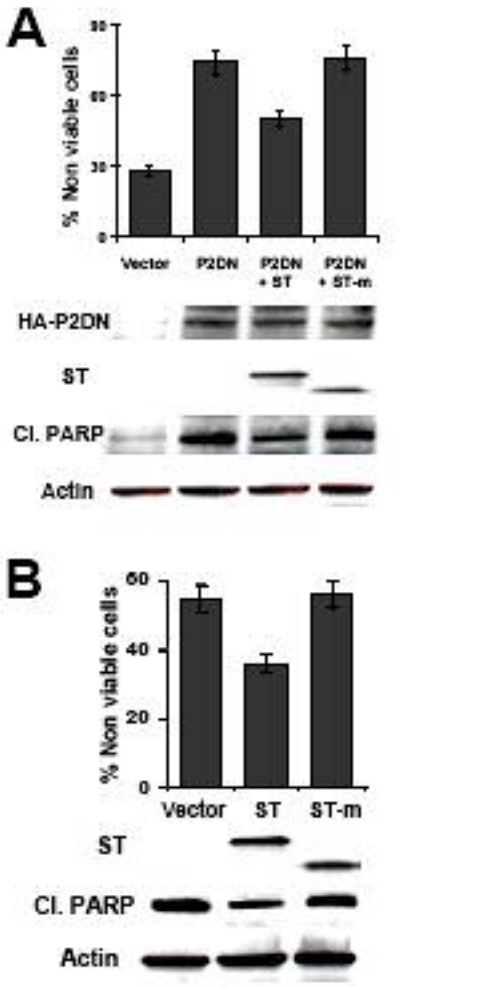


Fig4. Inhibition of PP2A restores the survival signals in MDA-MB-231 cells lost upon inhibiting either mTOR or PLD. *A,* PP2A activity in MDA-MB-231 cells was determined as Fig. 1. Fostriecin (FOS) (5 μM) and Cyclosporin A (CsA) (1 μM) were added where indicated 16 hr prior to assessing PP2A activity. The PP2A activity was normalized to the PP2A activity in the DMSO vehicle control, which was given a value of 1. Error bars represent the standard deviation for triplicate samples from a

representative experiment repeated 3 times. *B,* MDA-MB-231 cells were placed in serum free media along with rapamycin (Rap) (20 μM), FOS (5 μM), CsA (1 μM) or the DMSO vehicle as indicated. 16 hr later, cell viability was determined by trypan blue exclusion as described in Experimental Procedures. PARP cleavage was determined by Western blot analysis using an antibody that recognizes cleaved PARP (Cl PARP). Actin loading controls were performed by Western blot. The experiment is representative of one repeated 3 times. *C,* 231-v and 231-P2DN cells were placed in serum free along with FOS (5 μM), CsA (1 μM) or the control (Con) DMSO vehicle as indicated. 24 hr later, cell viability and PARP cleavage was determined as in *B*. The experiment is representative of one repeated 3 times.

Fig5. SV40 small t-antigen restores survival of MDA-MB-231 cells lost by suppression of PLD activity. *A,* MDA-MB-231 cells were placed in serum free media and then co-transfected with either vector alone, vector expressing the dominant negative PLD2 (P2DN), P2DN and vector expressing SV40 small T antigen (ST), and P2DN and SV40 small T antigen mutant lacking the PP2A binding site (ST-m). 24 hours later, cell viability and PARP cleavage were determined as in Fig. 4. The levels of dominant negative PLD2 and small t-antigen expression were determined by Western blot. The dominant negative PLD2 was Flu-tagged (Y11) and an antibody raised against the Flu-tag was used to distinguish from endogenous PLD2. *B,* The experiment performed in *A* was repeated using MDA-MB-231 cells that stably express dominant negative PLD2, except that the transfections were done without the PLD2 vectors. Experiments are representative of those repeated 2 times.

Suppression of mTOR or PLD inhibits phosphorylation of BAD at Ser112

BAD, a pro-apoptotic molecule of the Bcl2 family of apoptosis regulators, is regulated by reversible phosphorylation (Zha *et al* 1996), and is a target of survival signals (Downward *et al* 2004). Dephosphorylation of BAD at Ser112 by PP2A has been shown to be critical for the pro-apoptotic effects of BAD (Chiang *et al* 2001, 2003). If PLD is suppressing PP2A in an mTOR-dependent manner, then suppression of PLD signaling should suppress phosphorylation of BAD at Ser112. MDA-MB-231 cells were treated with either rapamycin or dominant negative PLD2. As shown, in Fig. 6A, rapamycin suppressed the phosphorylation of PP2A at Ser112. The suppression of BAD phosphorylation was reversed by the PP2A inhibitor FOS, indicating that the reduced phosphorylation of BAD in the presence of rapamycin was due to PP2A. We also examined the effect of the dominant negative PLD2 on BAD phosphorylation. The vector expressing the dominant negative PLD2 mutant was transiently transfected into MDA-MB-231 cells and BAD phosphorylation was investigated 24 hr later. As shown in Fig. 6B, the dominant negative PLD2 also suppressed BAD phosphorylation at Ser112. If the small t-antigen expressing vector was co-transfected, the effects of the dominant negative PLD2 were reversed. The reversal was not observed if the small t mutant that does not bind PP2A was used. These data further indicate that the mTOR-dependent signals generated by PLD involve PP2A. These data also suggest that the survival signals generated by PLD involve suppression of BAD dephosphorylation by PP2A.

Fig6

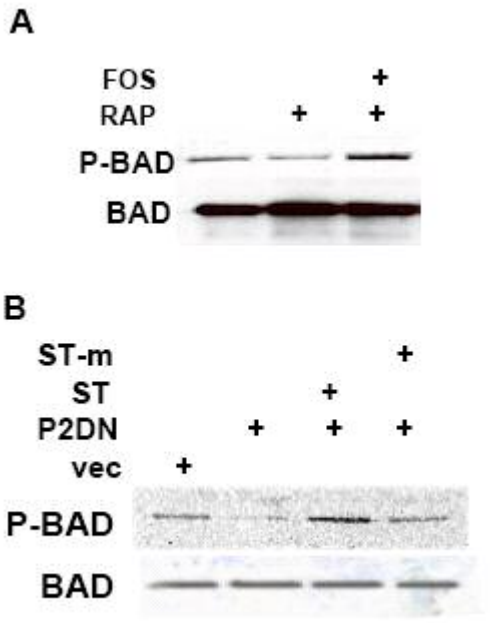


Fig6. Suppression of PLD or mTOR reduces phosphorylation of BAD at Ser112. *A,* MDA-MB-231 cells were treated with rapamycin (RAP) (20 μM) and fostreicin (FOS) (5 μM) as indicated. Two hr later, the cells were harvested and analyzed for phosphorylated BAD and total BAD by Western blot analysis. *B,* MDA-MB-231 cells were transiently transfected with an empty vector control (vec) or vectors expressing a dominant negative PLD2 mutant (P2DN), SV40 small t antigen (ST), or a mutant of small t that does not bind PP2A ST-m – where indicated. 24 hr later, the cells were harvested and analyzed for phosphorylated BAD and total BAD as in *A*. The experiment is representative of one repeated 2 times.

DISCUSSION

The generation of survival signals in an emerging tumor is critical if cells are to escape from the normal constraints built in to prevent unwanted proliferation (Hanahan *et al* 2000). During the past several years it has become apparent that mTOR is a critical target of survival signals and for progression through cell cycle checkpoints (Hahn 2002, Ruggero 2003, Foster *et al* 2004). The demonstration of a phosphatidic acid requirement for mTOR (Fang *et al* 2001) implicated PLD activity in the generation of mTOR-mediated survival signals. Consistent with a role for PLD in mTOR-mediated survival signals, elevated PLD activity in the human breast cancer cell line MDA-MB-231 cells provided a rapamycin-sensitive survival signal (Chen *et al* 2003). Moreover, elevated PLD activity has been implicated in breast, kidney, gastric and colon cancer (see Foster *et al* 2003, for review), indicating that PLD plays a role in human cancer. In this report we have shown that the elevated PLD activity in the human breast cancer cell line MDA-MB-231 cells leads to an mTOR-dependent suppression of PP2A that is critical for suppressing apoptosis.

The requirement of SV40 small t-antigen for the transformation of human cells with SV40 early region genes (Hahn *et al* 2002) underscores the importance of targeting PP2A in human cancer. We recently reported that PLD activity suppresses the induction of p53 (Hui *et al* 2004), which is also targeted by SV40 early region genes in the transformation of human cells (Hahn *et al* 1999, 2002). The data presented here are consistent with a model where the elevation of PLD activity suppresses apoptosis by facilitating progression through the same cell cycle checkpoints overcome by SV40 early

region genes. Consistent with this hypothesis, PLD, like SV40 early region genes, cooperates with signaling oncogenes to transform rat fibroblasts in culture (Hahn 1999, 2002, Lu 2000, Joseph *et al* 2001).

Recent studies have implicated dysregulation of translational control in a number of human cancers including breast (Antony 1996, Polunovsky 2000, Ruggero *et al* 2003). Increased expression of 4E-BP1, which inhibits eIF4E, was shown to revert the malignant phenotype of transformed rodent fibroblasts (Polunovsky *et al* 2000). Moreover, elevated expression of eIF4E partially rescued rapamycin-inhibited G1-phase progression (Fingar *et al* 2004), indicating that mTOR effects on cell cycle progression are mediated by a 4E-BP1 suppression of eIF4E. We demonstrated here that PP2A is associated with 4E-BP1 in MCF7 cells and that this association is disrupted in a rapamycin-dependent manner with elevated PLD activity. Similarly, association between PP2A and 4E-BP1 in MDA-MB-231 cells was stimulated by suppression of PLD activity. Since hyperphosphorylated 4E-BP1 has a decreased affinity for eIF4E, the association between PP2A and 4E-BP1 would likely result in the dephosphorylation of 4E-BP1 and the sequestering of eIF4E. Thus, the dissociation of PP2A from 4E-BP1 induced by PLD activity should enhance the release of eIF4E form the inhibitory constraints of 4E-BP1 and stimulate the initiation of translation. The ability of PLD to stimulate translation is consistent with the emerging paradigm that translation contributes to the survival signals.

PP2A has also been shown interact with and dephosphorylate the pro-apoptotic Bcl family protein BAD at Ser112 (Chiang *et al* 2001, 2003). This dephosphorylation is necessary for the pro-apoptotic effects of BAD (38). Data presented here indicate that the suppression of PP2A by PLD also reduces phosphorylation of BAD at Ser112. The

ability of PLD to prevent the dephosphorylation of BAD likely contributes to the ability of PLD to suppress apoptosis and underscores the critical importance of targeting PP2A in survival signals.

The data presented here provide further evidence on the ability of elevated PLD activity in human cancer cells to interfere with signals that regulate cell cycle progression and apoptosis. The data reported here reveal an mTOR dependent suppression of PP2A activity, which is apparently critical for the transformation of human cells (Chen *et al* 2004). PLD also stimulated the dissociation of PP2A from the mTOR substrates S6-kinase and 4E-BP1, and suppressed the dephosphorylation of BAD at a PP2A site. Since PLD activity is elevated in a large number of human cancers (Foster *et al* 2003) and the suppression of PP2A is apparently critical for the transformation of human cells (Chen *et al* 2003, 2004), it is likely that the elevated PLD activity in human cancer is a critical component of tumor progression. A model for the mTOR-dependent targeting of PP2A by PLD-generated survival signals is shown in Fig. 7.

Fig7

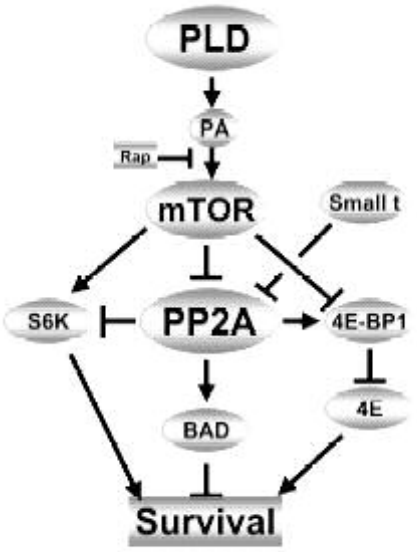


Fig7. Model for targeting of PP2A by PLD-generated survival signals. PLD generates phosphatidic acid (*PA*) from phosphatidylcholine, which can then stimulate mTOR. Activated mTOR could then phosphorylate PP2A and suppress its activity. This leads to dissociation of PP2A from the mTOR substrates S6-kinase and 4E-BP1. The suppression of PP2A also suppressed dephosphorylation of BAD at the PP2A site Ser-112. PP2A is also suppressed by SV40 small t-antigen, which is critical for the transformation of human cells.

CHAPTER V

Cooperation between Gain-of-Function p53 Mutants and Phospholipase D for Survival of Human Breast Cancer Cells

INTRODUCTION

Mutations in the p53 tumor suppressor gene are observed in greater than 50% of all human cancers (Hainaut *et al* 2000). However, unlike other tumor suppressor genes such as Rb and APC, over 85% of the mutations in the p53 gene are missence, rather than truncation or deletion mutants (Hinds 1990, Hollstein 1994 and Hainaut *et al* 2000). The observation that other tumor suppressor genes are commonly deleted in tumors leading to loss-of-function phenotypes suggests that the low incidence of deletion mutants for p53 in human cancer reflects a positive selection for the missence mutations in p53. Moreover, mutant p53 in human cancer is commonly expressed at high levels and is more stable than wild type p53 (Midgley 1997, Peng 2001a, b and Asher 2003). These observations suggest that there is positive selection for mutations to p53 that contribute to tumorigenesis. Initially, p53 was thought to be an oncogene because it was over-expressed in many cancers and p53 genes from transformed cells was able to act like a dominant oncogene in transformation assays (Eliyahu 1984, Jenkins 1984, Parada 1984 and Wolf *et al* 1984). One mechanism whereby mutant p53 can act like a dominant oncogene is through a "dominant negative" effect by interacting with and inactivating wild type p53 (Blagosklonny *et al* 2000). However, wild type/mutant p53 genotypes in human cancer are very rare (Blagosklonny *et al* 2000). p53 mutants have been shown to confer tumorigenic properties to cells lacking wild type p53 (Ditter 1993, Hsiao 1994, Lanyi 1998, Scian *et al* 2004), indicating that the ability of p53 genes to contribute cell transformation goes beyond dominant negative effects. Moreover, tumor-derived p53 mutants have been shown to activate promoters not activated by wild type p53 including

those for the EGF receptor (Deb *et al* 1994), c-Myc (Frazier *et al* 1998), c-Fos (Preuss *et al* 2000), and others (Yang 1999, Van Oijen 2000 and Cadwell 2001). Expression of a p53 with a mutation at any one of the four amino acid positions 175, 248, 273, or 281 was shown to correlate with increased PCNA promoter activity (2- to 11-fold) (Deb *et al* 1992). Mutations at 175, 248 and 273 are the 3 most common mutations in p53 and constitute close to 20% of the mutations seen in p53 (Van Oijen *et al* 2000).

In an emerging tumor, cells undergo the stress of nutrient, oxygen, and growth factor deprivation prior to angiogenesis, which will ordinarily lead to apoptosis. Therefore, it is critical that tumor cells acquire the capability of suppressing default apoptotic signals to insure survival (Hanahan *et al* 2000). MDA-MB-231 human breast cancer cells have high levels of phospholipase D (PLD) activity (Chen 2003, Zhong *et al* 2003) that generates a survival signal that suppresses apoptosis when cells are subjected to the stress of serum withdrawal (Chen 2003, Foster 2003 and Foster *et al* 2004). MDA-MB-231 cells, like many cancer cells express high levels of a mutant p53. The mutation to p53 in MDA-MB-231 cells is an R→K mutation at position 280 (Olivier *et al* 2002). This site is adjacent to the well-characterized p53 gain-of-function mutation at position 281 (Lanyi 1998 and Atema *et al* 2002) and has also been observed in nasopharyngeal carcinoma (Sun *et al* 1992). We have examined whether the mutant p53 in MDA-MB-231 cells is critical for the survival of these cells. We report here that mutant p53 is required for the survival signals generated by PLD in MDA-MB-231 breast cancer cells subjected to the stress of serum withdrawal. Mutant p53 was also required for the survival of BT-549 breast cancer cells, which also have elevated levels of PLD activity.

RESULTS

Elevated expression of p53 in MDA-MB-231 cells is dependent upon PLD.

MDA-MB-231 cells have a mutant p53 (Olivier *et al* 2002), whereas MCF7 have wild type p53 (Lu *et al* 2001). As shown in Fig. 1A, MDA-MB-231 cells express very high levels of p53 relative to MCF7 cells. We previously reported that elevating PLD activity in MCF7 cells suppresses p53 stabilization (Hui *et al* 2004). Since MDA-MB-231 cells have 10-fold greater PLD activity than MCF7 cells (Chen *et al* 2003, 2005), we examined the effect of suppressing PLD activity on p53 expression by introducing siRNA for PLD2 into MDA-MB-231 cells. As shown Fig. 1B, PLD2 siRNA suppressed PLD activity in MDA-MB-231 by close to 80%. Surprisingly, PLD2 siRNA also reduced p53 levels in the MDA-MB-231 cells (Fig. 1C). Thus, PLD activity is required at least in part for the elevated level of the p53 mutant in MDA-MB-231 cells.

Fig1.

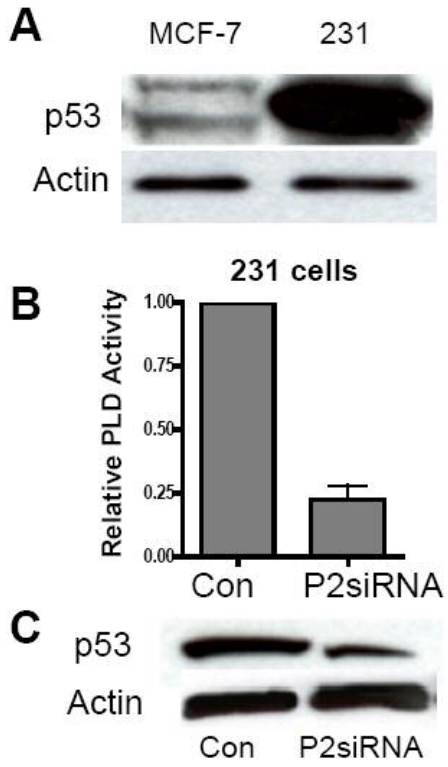


Fig1. Elevated expression of p53 in MDA-MB-231 cells is dependent upon PLD.

(A) The level of p53 protein in MCF7 and MDA-MB-231 (231) cells was determined by Western blot analysis. To control for loading, blots were re-probed with an anti-actin antibody. *(B)* MDA-MB-231 cells (231 cells) were transfected with either control (Con) GAPDH or PLD2 siRNA and PLD activity was determined using the transphosphatidylation reaction as described in the methods section. The PLD activity in the cells treated with the PLD2 siRNA was normalized to the control, which was given a value of one. Error bars represent the standard deviation for duplicate samples. *(C)* MDA-MB-231 cells were transfected with either control (Con) GAPDH or PLD2 siRNA

and the level of p53 was determined 72 hr later as in A. Experiments in *A, B*, and *C* are representative of experiments repeated at least two times.

Elevated p53 in MDA-MB-231 cells is dependent upon MAP kinase, but not mTOR. We reported previously that survival signals generated by PLD have requirements for both mTOR (Chen *et al* 2005) and MAP kinase (Hui *et al* 2004). The PLD metabolite phosphatidic acid has been reported to activate mTOR in a manner that is competitive with rapamycin (Fang 2001, Chen 2003, Foster *et al* 2004). We therefore examined the effect of rapamycin on p53 levels in MDA-MB-231 cells. As shown in Fig. 2, the level of p53 in MDA-MB-231 cells was insensitive to rapamycin at concentrations that inhibited the phosphorylation of the mTOR substrate ribosomal subunit S6 kinase. Thus, the PLD dependent expression of p53 is apparently independent of mTOR. We next examined the effect of inhibiting MEK, the kinase that phosphorylates and activates MAP kinase. As shown in Fig. 2B, the MEK inhibitor U0126 suppressed both p53 expression and the phosphorylation of MAP kinase. The data in Fig. 2 indicate that the PLD-dependent expression of p53 in MDA-MB-231 cells involves the MAP kinase pathway, but not mTOR.

Fig2.

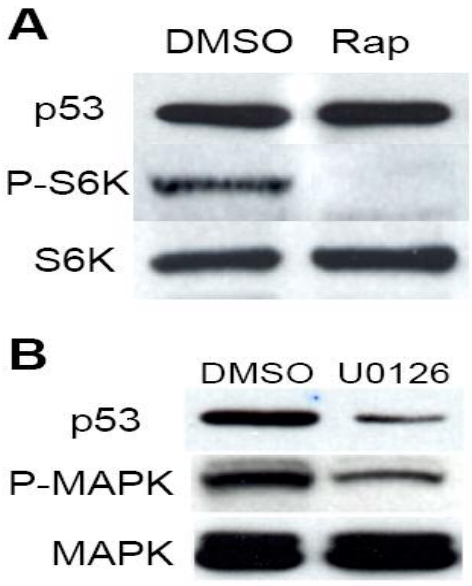


Fig2. Elevated expression of p53 in MDA-MB-231 cells is independent of mTOR and dependent on MAP kinase. *(A)* MDA-MB-231 cells were treated with either DMSO solvent (0.1%) or rapamycin (Rap) (20 μM) for 1 hr. The levels of p53, phosphorylated S6 kinase (S6K) and S6K were then determined by Western blot analysis. *(B)* MDA-MB-231 cells were treated with either solvent (DMSO) or the MAP kinase inhibitor U0126 (20 μM) for 1 hr. The levels of p53, phosphorylated MAP kinase (P-MAPK) and MAPK were then determined by Western blot analysis. Experiments shown in *A* and *B* are representative of experiments repeated at least two times.

Suppression of PLD or MAP kinase signaling increases the turnover of p53.

The level of p53 is commonly regulated at the level of stabilization. Continuously expressed p53 is degraded by the proteasome after ubiquitination by the E3 ubiquitin ligase HDM2 (Moll *et al* 2003). To examine whether PLD signaling impacted on the stability of p53, we examined the level p53 protein in cells treated with cyclohexamide to inhibit the synthesis of new p53 protein. As shown in Fig. 3A, the mutant p53 protein in MDA-MB-231 cells was relatively stable compared with the wild type p53 in MCF7 cells over a 90 min time course of cyclohexamide treatment. However, if the cells were treated with PLD2 siRNA (Fig. 3B) or U0126 (Fig. 3C), the stability of p53 was reduced. Thus, the increased stability of p53 in MDA-MB-231 cells is enhanced by PLD and MAP kinase signaling.

We next examined the impact of suppressing PLD and MAP kinase on the association between p53 and HDM2 in MDA-MB-231 cells. As shown in Fig. 4A, suppression of PLD activity with PLD2 siRNA led to an increased association between p53 and HDM2 as indicated by increased co-immunoprecipitation of p53 and HDM2. Similarly, suppression of MAP kinase activation with U0126 also increased the association between p53 and HDM2 (Fig. 4B). This observation is consistent with the increased turnover of p53 observed when either PLD or MEK was suppressed. We reported previously that PLD activity increases MDM2 levels in rat fibroblasts (Hui *et al* 2004). We therefore examined the effect of PLD2 siRNA and U0126 on the level of HDM2 in MDA-MB-231 cells. Consistent with our previous study, suppression of PLD activity (Fig. 4C) or MAP kinase activation (Fig. 4D) led to reduced HDM2 levels. Thus, interestingly, the increased association between p53 and HDM2 upon suppression of PLD

or MAP kinase occurs with reduced levels of HDM2. These data indicate that elevated PLD and MAP kinase activity suppress interaction between mutant p53 and HDM2 in MDA-mb-231 cells.

Fig3.

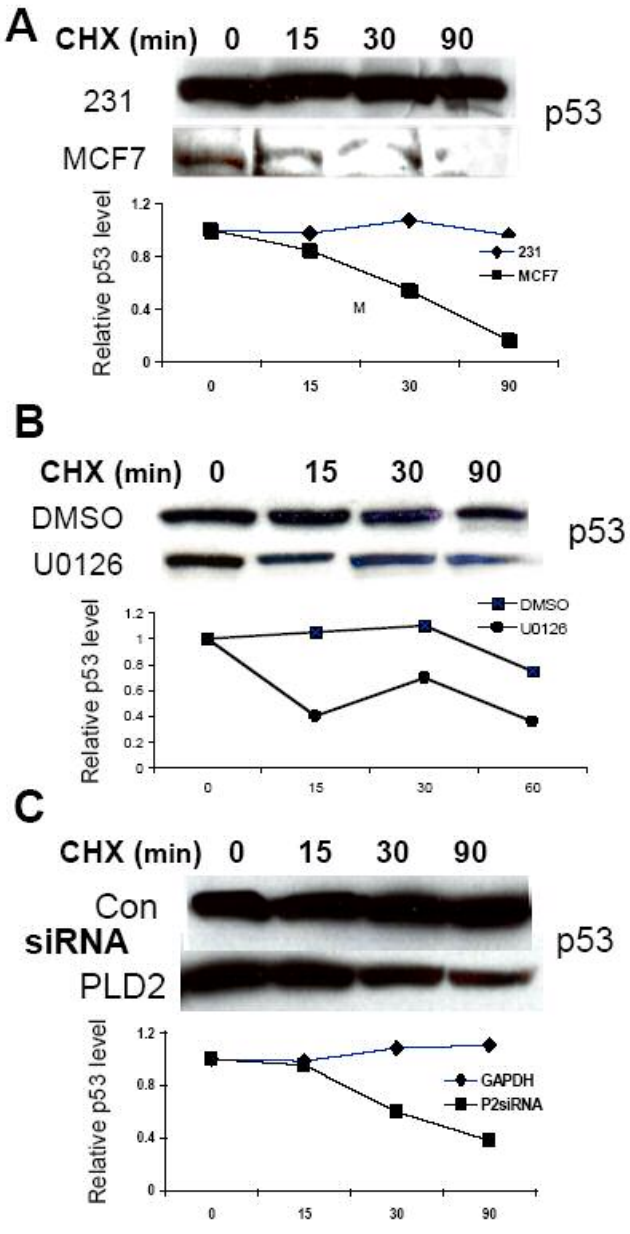


Fig3. Suppression of PLD or MAP kinase signaling increases the turnover of p53. *(A)* MCF7 and MDA-MB-231 cells were treated with cyclohexamide (80 μg/ml) for the indicated times. Cells were then harvested and p53 levels were determined by Western blot analysis. In the lower panel is a representation of the data in the upper panel from densitometer tracings of the autoradiogram in the upper panel. *(B)* MDA-MB-231 cells were transfected with either PLD2 or GAPDH control siRNA as in Fig. 1. 72 hr later the cells were treated with cyclohexamide and p53 levels were determined as in *A*. *(C)* MDA-MB-231 cells were treated with either U0126 or DMSO control for 1 hr. The cells were treated with cyclohexamide and p53 levels were determined as in *A*. Experiments shown are representative of experiments repeated at least two times.

Fig4.

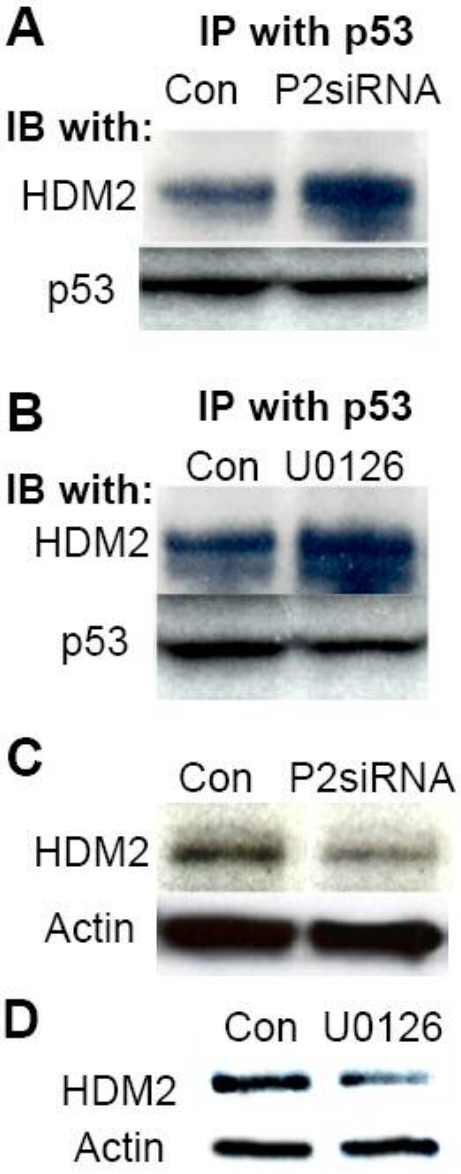

A
IP with p53
Con
P2siRNA
IB with:
HDM2
p53
B
IP with p53
Con
U0126
IB with:
HDM2
p53
C
Con
P2siRNA
HDM2
Actin
D
Con
U0126
HDM2
Actin

Fig4. Suppression of PLD or MAP kinase signaling increases the association of p53 and HDM2 in MDA-MB-231 cells. *(A)* MDA-MB-231 cells were transfected with either PLD2 or GAPDH control siRNA as in Fig. 1. 72 hr later, the cells were lysed and the lysates were immunoprecipited (IP) with an anti-p53 antibody. The immunoprecipitates were then subjected to Western blot analysis (IB) with either HDM2 or p53 antibodies as indicated. *(B)* MDA-MB-231 cells were treated with either U0126 or DMSO control for 1 hr. The cells were then lysed and the lysates were immunoprecipited (IP) with an anti-p53 antibody and the immunoprecipitates were then subjected to Western blot analysis (IB) with either HDM2 or p53 antibodies as in *A*. *(C)* MDA-MB-231 cells were transfected with either PLD2 or GAPDH control siRNA as in A and HDM2 levels were determined by Western blot. *(D)* MDA-MB-231 cells were treated with either U0126 or DMSO control for 1 hr and HDM2 levels were determined as in *C*. Experiments shown are representative of experiments repeated at least two times.

Survival signals generated by PLD are dependent upon mutant p53.

While many p53 mutations result in "loss of function" that eliminate the tumor suppressing effects of p53 (Lavine *et al* 1991), the most common p53 mutations are "gain-of-function" mutations that can actually enhance tumorigenesis when introduced into other cells (Zambetti 1993, Blandino 1999, Van Oijen 2000 and Cadwell *et al* 2001). Since PLD provides a survival signal in MDA-MB-231 cells that suppresses apoptosis when cells are subjected to serum withdrawal (Zhong 2003, Chen 2005), we investigated whether the PLD-dependent p53 expression in MDA-MB-231 is critical for the survival of these cells subjected to serum withdrawal. We first examined the effect of suppressing

PLD activity with PLD2 siRNA on cell viability and PARP cleavage in MDA-MB-231 cells subjected to serum withdrawal. As shown in Fig. 5A, PLD2 siRNA reduced cell viability and increased PARP cleavage indicating that the loss in cell viability was due to apoptosis. Similarly, the MEK inhibitor U0126 also reduced cell viability and increased PARP cleavage in the MDA-MB-231 cells deprived of serum (Fig. 5B). We then examined the impact of p53 siRNA on the survival of MDA-MB-231 cells, and as shown in Fig. 5C, the p53 siRNA strongly suppressed p53 expression and induced apoptosis in serum starved MDA-MB-231 cells as indicated by loss of cell viability and increased PARP cleavage. These data indicate that the mutant p53 expressed in MDA-MB-231 cells is required for the survival signals generated by PLD.

Fig5.

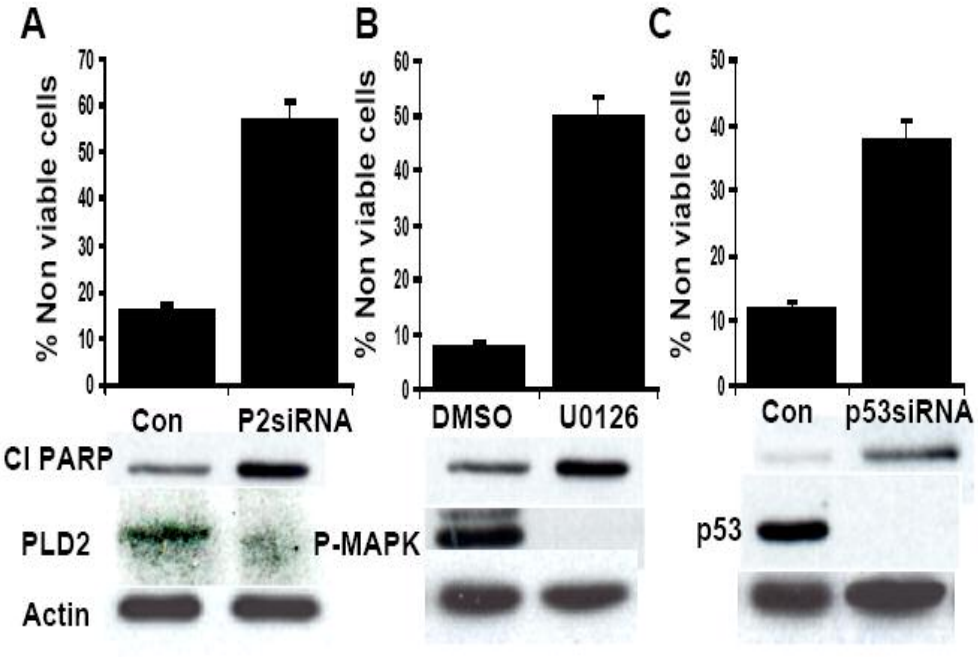


Fig5. Surrvival signals generated by PLD are dependent upon PLD and transformation pon mutant p53. *(A)* MDA-MB-231 cells were plated in DMEM with 10% serum for 24 hr. The cells were then transfected with either control GAPDH or PLD2 siRNA. 24 hr later the cells were shifted to fresh media containing 0% serum for 48 hr, at which time the percentage of non-viable cells was determined by trypan blue exclusion. At this time cells were also examined for the level of cleaved PARP (Cl PARP) and PLD2 protein using Western blot analysis. Blots were stripped and reprobed with an antibody to actin to control for loading. *(B)* MDA-MB-231 cells were plated in DMEM with 10% serum for 24 hr. The cells were then shifted to fresh media containing 0% serum for 16 hr in the presence of either U0126 (20 μM) or the solvent DMSO, at which time the percentage of non-viable cells and the level of cleaved PARP (Cl PARP) was determined as in *A*. The effect of U0126 on MAP kinase phosphorylation is also shown. *(C)* MDA-MB-231 cells were plated in DMEM with 10% serum for 24 hr. The cells were then transfected with either control GAPDH or p53 siRNA. 24 later the cells were shifted to fresh media containing 0% serum for 48 hr, at which time the percentage of non-viable cells and the level of cleaved PARP (Cl PARP) was determined as in *A* and *B*. The effect of p53 siRNA on p53 protein levels is also shown. The experiments shown in *A, B*, and *C* are representative of at least two independent experiments.

Suppression of p53 expression in other breast cancer cells with gain-of-function. The data obtained with the MDA-MB-231 cells demonstrated that the p53 mutant in these cells was critical for the ability of these cells to survive in the absence of serum growth factors. We next examined whether p53 gain-of-function mutants in other breast

cancer cells was critical for survival. BT549 (R249S) and SKBR3 (R175H) cells express well-characterized p53 gain-of-function mutants (Van Oijen 2000, Tsang *et al* 2005). T47D (L194F) has a dominant negative phenotype that suppresses p21 expression (Epstein *et al* 1998). All three cell lines expressed p53 levels similar to that observed for the MDA-MB-231 cells (Fig. 6A). The level of PLD activity was also examined in these cells and as shown in Fig. 6B, it can be seen that there is elevated PLD activity in the BT549, but not the T47D or SKBR3 cells. We next examined the effect of depleting p53 on the ability to survive in the absence of serum. p53 siRNA was introduced into the BT549, T47D, and SKBR3 cells and the impact on cell viability and PARP cleavage was evaluated. As shown in Fig. 6C, suppression of p53 expression resulted in cell death and PARP cleavage in the BT549 cells, which have high levels of PLD activity, but had no effect on the survival of the T47D or SKBR3 cells, which have very low levels of PLD activity. These data indicate that mutant p53 is required for the survival of some but not all breast cancer cell lines with elevated expression of p53 mutants. The data also suggest a selection for the effect of mutant p53 in cells with elevated PLD activity.

Fig6.

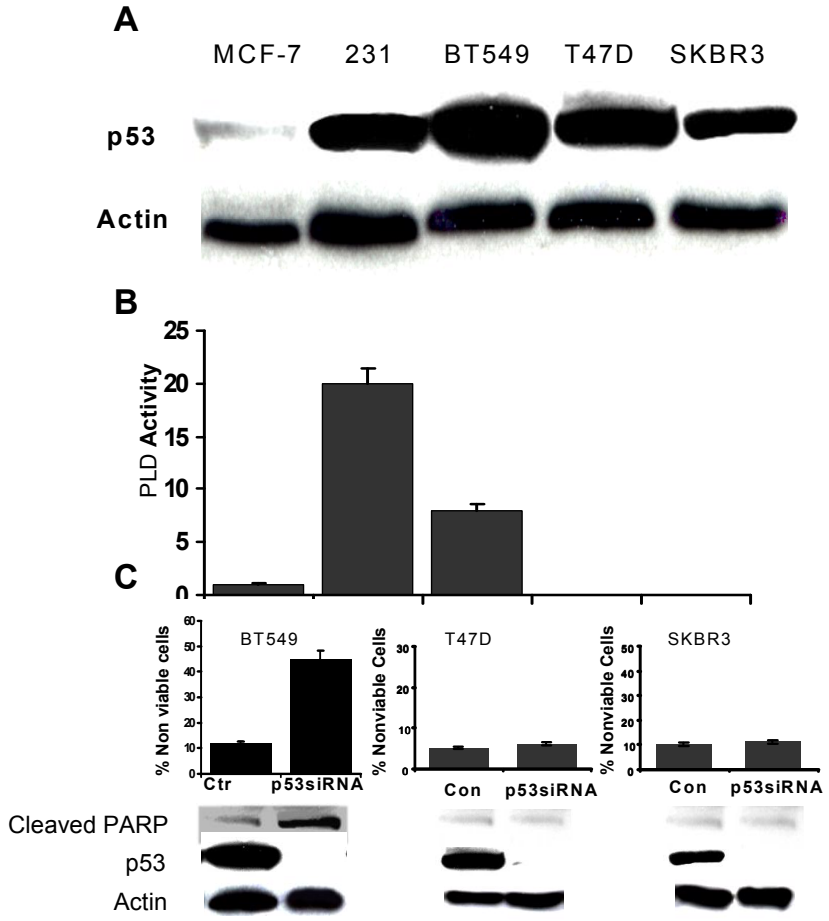


Fig6. Effect of p53 siRNA on survival of BT549, T47D, and SKBR3 breast cancer cells. (*A*) p53 levels in MCF-7, MDA-MB-231, BT549, T47D, and SKBR3 was evaluated by Western blot as in Fig. 1. (*B*) The PLD activity in the breast cancer cells used in *A* was determined as in Fig. 1. (*C*) The effect of depleting cells of p53 with siRNA was investigated as described in Fig. 5. Experiments shown are representative of two independent experiments.

DISCUSSION

In this report we have demonstrated that survival signals generated by PLD in MDA-MB-231 cells require expression of mutant p53. PLD increased the half-life of mutant p53 leading to increased levels of p53 in these cells. These data indica te that the mutant p53 in MDA-MB-231 cells is critical for the survival of these cells under the stress of serum withdrawal. p53 mutants have been reported to act as dominant negative mutants to suppress wild type p53, however there is no wild type p53 in MDA-MB-231 cells (Katayose *et al* 1995), indicating that the impact mutant p53 upon the survival of MDA-MB-231 cells is not due to interfering with wild type p53. Mutant p53 expressed in BT549 cells, which have elevated levels of PLD activity, was also critical for survival in the absence of serum. In contrast, SKBR3 cells, which have a gain-of-function p53 mutation but do not have elevated PLD activity, did not require mutant p53 expression for survival. A dominant negative p53 mutation in T47D cells was similarly not required for survival. These data reveal that p53 gain-of-function mutations are capable of providing a critical selective advantage to breast cancer cells by suppressing cell death – especially those with elevated PLD activity. These data are largely consistent with a very recent report indicating that suppression of mutant p53 in human cancer cells reduced malignancy (Blagosklonny *et al* 2000) and support a model whereby p53 gain-of-function mutations provide a selective advantage during early stages of tumorigenesis where suppression of default apoptotic signals is critical.

Survival signals generated by PLD are dependent on mTOR (Chen *et al* 2005). The effect of PLD on p53 expression in MDA-MB-231 cells was independent of mTOR

suggesting that activation of mTOR by PLD likely has additional effects upon survival. The effect of PLD on p53 expression in MDA-MB-231 cells was dependent on the MAP kinase signaling pathway. We demonstrated previously that PLD stimulated MAP kinase phosphorylation by enhancing endocytosis of the epidermal growth factor (EGF) receptor (Shen *et al* 2001), which is required for the phosphorylation of MAP kinase in response to EGF (Kranenburg 1999, Shen *et al* 2001). In this regard it is of interest that the EGF receptor is over-expressed in MDA-MB-231 cells (Moasser *et al* 2001), and that PLD can cooperate with elevated expression of the EGF receptor to transform cells in culture (Lu 2000, Joseph 2001). In addition, MAP kinase is highly activated in MDA-MB-231 cells (Ogata *et al* 2001). Thus, the dependency of MDA-MB-231 cells on mutant p53 for survival may involve activation of the MAP kinase pathway by enhancing the effects of elevated expression of the EGF receptor. However PLD likely provides additional survival protection through mTOR activation.

The ability of PLD to increase the level of p53 was quite surprising in that our previous study indicated that elevated PLD activity suppresses DNA damage-induced increases in wild type p53 (Hui *et al* 2004). The mechanism for this suppression was apparently due to increased expression of HDM2 induced by PLD. Consistent with our previous report, suppression of PLD activity led to reduced levels of HDM2 in the MDA-MB-231 cells. In spite of the reduced level of HDM2 observed with PLD2 siRNA, there was increased association between p53 and HDM2. This indicated that elevated PLD activity in MDA-MB-231 was in some way suppressing the interaction between p53 and HDM2. Suppression of the p53 – HDM2 interaction was dependent upon MAP kinase, suggesting that phosphorylation may play a role. However, MAP kinase has not been

shown to phosphorylate p53 directly. Thus, at this point it is not clear how the p53 – HDM2 interaction is disrupted with elevated PLD activity, but it is clear that while elevated PLD activity can promote HDM2 levels, elevated PLD activity also suppresses that interaction between mutant p53 and HDM2.

p53 mutations are far and away the most common in human cancer with some estimates indicating that as many as 70% of human cancer having mutations in the p53 gene. While knocking out the tumor suppressing effects of p53 are critical for allowing passage through cell cycle checkpoints, the ability of point mutations in the p53 gene to produce p53 proteins with dominant gain-of-function oncogenic properties makes p53 a double edged sword. An early point mutation in p53 could lead to elevated expression of Myc and other proteins that could provide survival signals that suppress apoptosis in an emerging tumor prior to vascularization. Loss of heterozygosity for the p53 gene, which generally late in tumorigenesis (Kinzler *et al* 1996), would further abrogate any remaining tumor suppressing effects of wild type p53 and enhance genomic instability by disabling cell cycle checkpoints. The data provided here demonstrate that in the human breast cancer cell line MDA-MB-231, p53 is required for survival under conditions of serum withdrawal - conditions similar to that in an emerging tumor before vascularization. A critical problem for tumorigenesis in cells with two wild type alleles is obtaining mutations to both genes in the same cells. The probability of generating mutations on both alleles is quite remote unless there is a selection for the first mutation. In the case of p53, the gain-of-function mutation – at least for MDA-MB-231 cells – is able to contribute to survival signals that are critical early in tumorigenesis to suppress default apoptotic programs. Once a dominant gain-of-function p53 mutation is selected for,

progression to loss of heterozygosity would be relatively common and generate a complete loss-of-function for the tumor suppressing gatekeeper function of p53 (Fig. 7). Elevated PLD activity could enhance the effectiveness of gain-of-function p53 mutations by increasing the stability of the mutant p53 relative to the wild type p53.

Fig7.

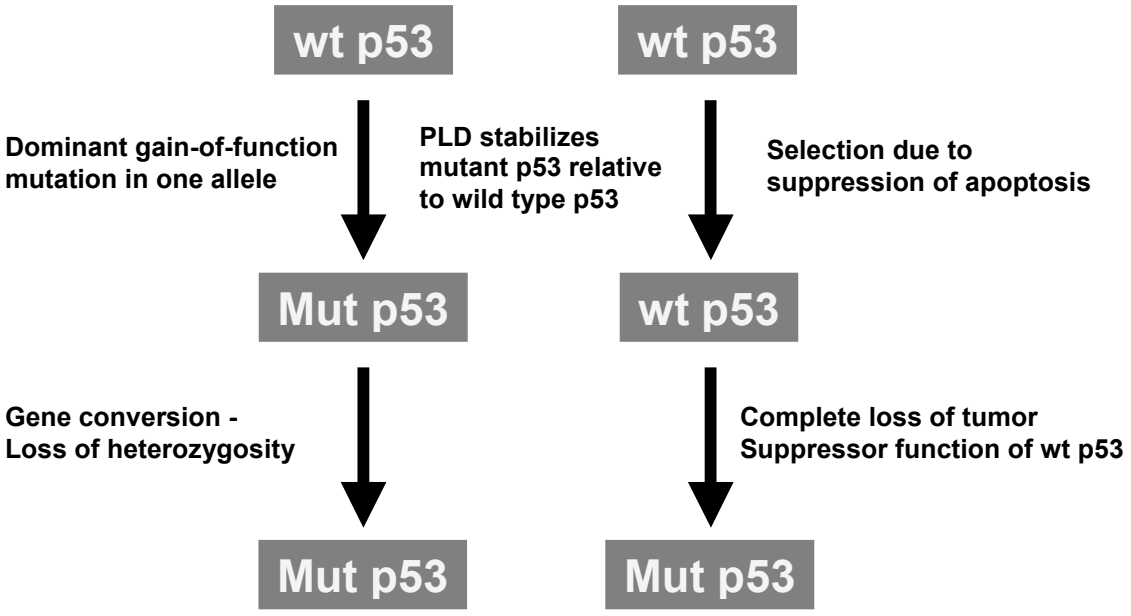


Fig7. Model for the selection of p53 gain-of-function mutants stabilized by PLD. Cells acquiring a gain-of-function mutation to p53 would be selected for because of the ability to suppress apoptosis during early stages of tumorigenesis. Since PLD is able to enhance the stability of mutant p53, cells with elevated PLD activity would have higher levels of mutant p53 relative to wild type p53. Gene conversion leading to loss of

heterozygosity would eliminate wild type p53 and any remaining tumor suppressor capability.

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