Lipase-Kinase Associations Involving PLD2, JAK3 and FES that Underlie Cancer Cell Proliferation and Invasion

Qing Ye

Wright State University

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LIPASE-KINASE ASSOCIATIONS INVOLVING PLD2, JAK3 AND FES THAT UNDERLIE CANCER CELL PROLIFERATION AND INVASION

A thesis submitted in partial fulfillment of the requirements for
the degree of Master of Science

By

QING YE

Wright State University
Dayton, Ohio

December, 2012
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Qing Ye ENTITLED Lipase-Kinase Associations Involving PLD2, JAK3 and FES that Underlie Cancer Cell Proliferation and Invasion BE ACCEPTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Julian Gomez-Cambronero, Ph.D
Thesis Director

Steven J. Berberich, Ph.D
Chair, Department of Biochemistry and Molecular Biology

Committee on Final Examination

Julian Gomez-Cambronero, Ph.D

Steven J. Berberich, Ph.D

Gerald M. Alter, Ph.D

Andrew Hsu, Ph.D, Dean, Graduate School
ABSTRACT

Phospholipase D (PLD) is an enzyme that breaks down phospholipids in the cell membrane. It has been suggested that PLD may play a role during cell proliferation and cell invasion of cancer cells. The objective of this thesis was to define new molecular signaling pathways in which PLD2 might be involved in terms of cell proliferation (first part) and cell invasion (second part). To this, I compared molecular and biochemical aspects between untransformed cell lines with highly invasive, transformed breast cancer cells.

In the first part, I investigated the interaction of two tyrosine kinases with PLD2 and the effect of such interaction on cell proliferation in the highly invasive MDA-MB-231 breast cancer cells. I found that the activities from the two kinases and from PLD2 are all augmented in the cancer cells while the protein levels are only relatively high in these cells. JAK3 upregulates PLD2 activity in MDA-MB-231 cells while inhibits PLD2 in the untransformed breast epithelial MCF10A cells. Fes interacts with PLD2 at distinct binding sites in MDA-MB-231 cells compared to MCF10A cells. I also found that phosphatidic acid (PA), the product of PLD enzymatic activity, enhances Fes activity in MDAMB-231 cells but not in MCF10A cells. Further, I demonstrate that the two tyrosine kinases JAK3 and Fes also interact with each other, with Fes having an inhibitory effect on JAK3 in MCF10A cells but not in cancer cells. The balanced PLD2 activity by JAK3, Fes and PLD2 interactions in the MCF10A cells keeps PLD2 at basal level that lead to normal cell growth. In contrast, in MDA-MB-231 cancer cells, the
effect of positive feedback among the three proteins and then enhancing effect of PA contribute to augmented proliferation.

In the second part of this thesis, I investigated the role of JAK3 on PLD2 activities during cell invasion in MDA-MB-231 cells using a small-molecule tyrosine kinase inhibitor, the flavonoid apigenin (4’,5,7-trihydroxyflavone), as well as RNA silencing. I found that both JAK3 and the tyrosine kinase, Epidermal Growth Factor Receptor (EGFR) are sensitive to the inhibitors and directly regulate PLD2 and induce the highly invasive phenotype of MDA-MB-231 cells. Further, serum-deprived cells in culture show an upregulated EGFR/JAK3/PLD2-PA system and are especially sensitive to a combination of JAK3 and PLD2 enzymatic activity inhibitors (i.e. 30 nM apigenin and 300 nM 5-Fluoro-2-Indolyl des-Chlorohalopemide [FIPI], respectively). Accordingly, cell invasion is enhanced by two kinases (EGFR and JAK3) and a phospholipase (PLD2) on multiple levels that provides regulatory flexibility and contribute to maximum aggressiveness in cell invasion in MDA-MB-231 breast cancer cells. This is especially important during serum starvation that coincides with migration of these cells to new locations.

In this thesis, I have demonstrated the existence of new signaling pathways: JAK3-Fes-PLD2 that plays a central role in cancer cell proliferation and EGFR-JAK3-PLD2 that facilitate cell invasion. These new pathways might be important to be used as therapeutic targets aimed at controlling the high proliferative and invasive phenotype of breast cancer cells.
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I. INTRODUCTION

Proliferation and Invasion of Breast Cancer

Breast cancer is one of the most common forms of cancer in the world, and it accounts for a quarter of all cancers in women. Breast cancer is particularly dangerous due to its ability to metastasize to other parts of the body, such as the lungs. Metastasis is the primary cause of mortality in patients afflicted with breast cancer (130). One of the means to abrogate the rampant metastasis is through disruption of cell proliferation, which is defined as the rate at which the cells multiply. In this study, I selected MDA-MB-231 cells as an ideal model due to their high rate of proliferation and PLD2’s impact on MDA-MB-231 cells (148). MDA-MB-231 cells possess the WNT7B oncogene, which leads to a disruptive expression of several genes (86). This knowledge coupled with PLD2’s uncontrolled activity suggested that deregulation possibly stemmed from proteins which affect PLD2 activity. By examining some of the root causes of uncontrolled PLD2 activity in these cells, it is hoped that I could extrapolate how and why PLD2 is activated constitutively in other cancers and develop therapies, which could target these pathways in cessation of tumor growth or metastasis.
II. LITERATURE REVIEW

History and Background of PLD

PLD was initially described in plants, specifically carrots. Hanahan and Cahikoff first characterized its enzymatic activity in homogenates from vegetables (44, 45). PLD was later characterized in viral (124), prokaryotic (93) and eukaryotic (106, 129) organisms, and its activity was found to be a consequence of applied cellular stimuli. Since the early 1980s when mammalian PLD activities were first described (46, 117, 131), numerous studies on cellular models as well as genetic models have been conducted to characterize the physiological and pathophysiological functions of PLD. Many findings have found an association of PLD with cancer (5, 52, 65, 135), neuronal pathophysiology (9, 132, 144), cardiovascular topics (137, 138, 140), spermatogenesis (69) and infectious diseases (55, 56). Therefore, further understanding and characterization of PLD is essential for a complete understanding the cause of these conditions.

PLD’s Enzymatic Activity

PLD employs water as a nucleophile to allow the breaking of the phosphodiester bond of the polar head of phosphatidylcholine (PC), which releases choline and membrane lipid phosphatidic acid (PA) (Figure 1). Apart from PC breakdown, PLD in the presence of alcohols like ethanol or butanol can catalyze the transphosphatidylation reaction resulting in the formation of phosphatidylalcohols (33, 113). The advantage of this reaction is that in the laboratory, tritium-labeled butanol would yield phosphatidylbutanol, if PLD is present in the samples, which is easily separated via thin layer chromatography and counted in a scintillation counter. This is the primary method in which I quantify PLD activity.
Figure 1. PLD chemical reaction. Upper half, PLD catalyzes the hydrolysis of PC to generate choline and PA. Lower half, PLD regulators and effectors.
Characteristics of PLD

The PLD superfamily is composed of a diverse group of proteins that include enzymes involved in phospholipid metabolism, a bacterial toxin, poxvirus envelop proteins and bacterial nucleases (Fig. 2). There are two classic mammalian isoforms of PLD, PLD1 (42) and PLD2 (26), which share 52% identity (Figure 2A). PLD1 is a 1072 amino acid, 120 kDa protein, while PLD2 is a 933 amino acid, 103 kDa protein. Both isoforms require phosphatidylinositol 4,5-bisphosphate (PIP2) as a co-factor for activity and release structurally identical PA species in mammalian cells.

PLD1 and PLD2 differ by their tissue and subcellular distribution. Human PLD1 is less abundant in comparison of high and variable levels of PLD2 mRNA in tissues (145). PLD2 is expressed more abundantly in blood leukocytes but it is expressed at low levels in liver and skeletal muscles (114). Both isoforms are found elevated in cancers with high tyrosine kinase expression, such as breast cancer tissues (135). Based on studies with PLD inhibitors it was found that cell proliferation in MDA-MB-231 cells rely more on PLD2 than on PLD1 (112). PLD1 is localized to secretory granules and lysosomes and is translocated to the plasma membrane upon cellular stimulation (78). In contrast, PLD2 localizes in sarcolemmal membranes, plasma membranes or the rims of the Golgi apparatus (109, 110), and it is constitutively expressed.

PLD1 contains a negatively regulatory loop region (66) that modifies PLD1’s activity upon caspase cleavage (105). The smaller PLD2 protein lacks the loop structure, which results in differential response to certain regulatory molecules such as GTPases. On the other hand, PLD1 and PLD2 share two highly conserved catalytic HKD sequences (HXKX₄DX₆), which are
Figure 2. PLD main characteristics. (A) The PLD superfamily include two classic PLD, PLD1 and PLD2, and non-classic PLDs. (B) Both PLD1 and PLD2 share two catalytic HKD domains, PH and PX domains. PLD2 does not bear a loop region that exists in PLD1. The “loop” is a non-conserved 120 amino acid stretch. Human PLD is expressed in blood leukocytes and in cancer cells. PLD1 has a constitutive low basal level of activities while PLD2 activity level is high.
critical for their lipase activity (Figure 2B.). Further, an association of N- and C-terminal region of PLD1 is required for catalytic activity. Other conserved features in the PLD family include a PIP$_2$ binding site, pleckstrin homology (PH) and phox homology (PX) domains. PIP$_2$ binding is required for the enzyme activity of PLD. The N-terminus of the PH domain contains two SH2-binding motifs and is critical for a PLD-aldolase interaction. The PX domain is also located at the N-terminus of PLD and is involved in intracellular protein localization and, binds to proteins or lipids. Both the PH and PX domains are important for the regulation of the lipase activity. Residues Y169 and Y179 (31) within the PX domain have been shown by our lab to be important for docking of SH2-containing proteins and Y415 has been shown to facilitate JAK3 binding (47) (Figure 3), which will be subjected to mutagenesis to generate PLD2 mutants in this thesis.

*Functions of PLD*

Our laboratory has sought to study the role of PLD2 in cancer cell invasion and metastasis. A participation of PLD in cell migration was initially documented in leukocytes (41, 82). Subsequently, it has been explained in other cell types that PLD2 induces cell migration and invasion in human cancer cells (148), phagocytes (16, 41, 82, 92), fibroblasts (72, 101) and epithelial cells (11, 87). S6K (72), Rac2 (17), tyrosine kinase Fer (53, 97) and phosphocofilin (43) are also involved in PLD2 mediated invasion.

PLD has also been documented to generate apoptotic signals in the signal transduction pathway (61, 79, 141) through increased expression of Bcl-2 and Bcl-xL (99). Inhibition of PLD signal transduction by famesol enhanced apoptosis (128). PLD also postpones or inhibits
apoptosis by attenuating the expression of the tumor suppressors early growth response-1 and phosphatase and the tensin homologue is deleted on chromosome 10 (67). In terms of cell proliferation and mitogenesis, PA, product of PLD’s cellular enzymatic activity, is associated with proliferation in osteoblastic (15) and pheochromocytoma PC12 cells (77). Elevated PLD expression by triptolide reduces proliferation in MDA-MB-231 breast cancer cells (60). It has been suggested that PLD2 is able to induce proliferation through its phosphorylation at residue Y179 (29, 81).

A number of other cellular events are also associated with PLD activity. Some early studies have established the role of PLD in membrane oxidative burst (NADPH oxidase or NOX2) (88) and transcription through transcription factor AP-1 and S1 (70). In T lymphoid Jurkat cells, PLD regulates transcription through transcription factor AP-1 and STAT (70). Other roles for PLD include mitochondrial fusion (22) and during development (75).

PLD also exerts its influence over cellular signaling primarily through generation of the phospholipid PA. PA is a typically located at the plasma membrane as an important part of the phospholipid bilayer (38). PA, as a secondary messenger, is implicated in a wide variety of biological processes.

Figure 3. Domain architecture of PLD2. PLD2 is composed of a pleckstrin homology (PH) domain, a phox domain, and two conserved HKD domains. Residues Y169 and Y179 are important for the docking of SH2-bearing proteins. Residue Y415 is essential for PLD.
signaling pathways due to its ability to influence the membrane localization of proteins (73, 147) and activate a number of cell signaling enzymes (32, 98, 139).

**Regulation of PLD2**

The role of PLD2 in signal transduction has been under investigation by many research groups. Two pathways have been established that regulate PLD enzymatic activity. One pathway depends on small GTPase proteins for signal transduction, since Arf, Rho and related proteins can regulate PLD activity (6, 12, 24, 102). The other pathways are via tyrosine kinases including growth factors/mitogens like EGF, PDGF, insulin and serum for PLD activation (2, 20, 50, 84, 119). PLD1 has low basal activity that is regulated by phosphoinositides, protein kinase C (PKC), small GTPases, like ARF, RhoA, Rac1 and Cdc42, as well as by protein phosphorylation. With the regulation of PLD1 well documented, investigation of the regulation of PLD2 is still an ongoing work by many researchers. It is known that PLD2 is subjected to comprehensive regulation for its activation. Upon activation, PLD can modulate downstream molecules during cell signaling through molecules such as mTOR (30), S6K (10) and Ras (7). Higher PLD2 activity has been found in a number of tumor cells and, therefore, this study focuses on PLD2 regulation by two tyrosine kinases, Fes and JAK3, in MDA-MB-231 human breast cancer cells.

**Feline Sarcoma Oncogene (Fes)**

Fes is a 93-kDa non-receptor protein tyrosine kinase encoded by the FES gene (76). It is involved in phosphorylation of several substrates which are typically found in macrophage function, including cytoskeletal rearrangement (59). Fes additionally phosphorylates substrates that lead directly to gene transcription (57). Fes possesses an SH2 domain and an F-BAR domain
interaction with a variety of consensus sequences containing phosphorylated tyrosine residues (35), (120). The SH2-mediated interaction with substrates (35, 120). Mutations of E469 and E472 or R483 in the SH2 domain have been shown to eliminate the binding ability of Fes (30, 35). The F-BAR and FX domains are involved in phospholipid binding, membrane localization and kinase activity. The sites of mutagenesis are indicated including E469 and E472 (89), important for ligand binding and K590 (13). These behaviors make Fes a prime candidate in terms of generation of cancerous phenotypes. Fes also demonstrates a unique property in that it is expressed differentially in cancer cells. Differentially expressed isoforms of Fes have been shown to cause leukemia via unregulated differentiation in certain cancers of the immune system (14). Janus Kinase 3 (JAK3)
JAK3 (Figure 5) is a kinase primarily involved in phagocytes and other cells of the innate immune system (125). While it is present in every cell at a basal level, it is typically expressed in an isoform that prevents kinase activity from becoming too high and is composed of seven Janus Homology (JH) domains with the motif in the JH4 domain, which has been shown to be integral in interacting with cytoskeletal proteins (90). Additionally, JAK3 has a catalytic kinase domain located in the JH1 domain at K855 (3). The N-terminal JH6 and JH7 domains regulate cytokine receptors and catalytic activity. Residues R402 and R403 in the JH4 SH2-like domain are important for ligand binding

**Figure 5. Domain architecture of JAK3.**

**Transformed and untransformed cell line models**

**MDA-MB-231 cells.** A number of cell line models are used as the primary model for transformed breast cancer cell lines. The MDA-MB-231 cell line was isolated from a female patient in 1973 (64, 130). This cell line is highly proliferative and metastatic (103). It functions as a valuable model for regulation of gene expression and cell proliferation in breast cancer. MDA-MB-231 cells, which have high levels of mutant p53, have high levels of PLD activity, which provides a survival signal in these cells when deprived of serum growth factors (51). This cell line is used as the primary model for transformed human cancer cells.
**MCF10A cells.** I chose the untransformed MCF10A cell line as a negative control for my study. The MCF10A cell line is derived from the MCF10 human breast epithelial cell line isolated from human fibrocystic mammary tissue (121). Subsequent culturing of MCF10 gave rise to MCF10A which is characterized as untransformed breast epithelium cells (71, 89, 121). MCF10A cells are positive for epithelial sialomucins, cytokeratins and milk fat globule antigen and exhibit three-dimensional growth in collagen and form domes in confluent cultures (40). This cell line is responsive to insulin, cholera enterotoxin, glucocorticoids, and epidermal growth factor (EGF) (71). Using electron microscopy, these cells display characteristics of luminal ductal cells but not of myoepithelial cells. Cell morphology is strongly affected by the calcium content of the medium. MCF10A cells also express breast specific antigens detected by positive reaction with MFA-breast and MC-5 monoclonal antibodies (40). This cell line expresses wild type p53 tumor suppressor.

**COS-7 cells.** COS-7 cells are derived from the CV1 cell line which was isolated in the 1960s from an African green monkey kidney (142). The COS-7 cell line was developed through CV1 transformation with a Simian Vacuolating Virus 40 (SV40), a virus that codes for the wild type virus T-antigen. COS-7 cells thus express the SV40 T-antigen and retain its susceptibility to SV40, which grows lytically (i.e. causing lysis) within the kidney cells (21, 108). In culture, COS-7 cells show adherent growth to glass and plastic surfaces. This cell line has been used extensively for transfection to produce recombinant proteins for molecular biology, biochemistry and cell biology experiments. This cell line expresses wild type p53 tumor suppressor.

**AML14.3D10 cells.** The AML14.3D10 cell line is a subclone from the AML14 human myeloid leukemia cell line in 1992 from acute myeloid leukemia (8, 100). The cells are eosinophil-like and are inducible for eosinophil differentiation to become either neutrophil-like
cells when exposed to all-trans retinoic acid (ATRA) or monocyte/macrophage with vitamin D or phorbol esters (8). As it is known that human neutrophils exhibit a regulated PLD activity, the further study of PLD activity and regulation of PLD in neutrophil-like cells from ATRA-treated AML14.3D10 cells is, therefore, examined in this thesis to compare to MDA-MB-231 cancer cells.

**MCF7 cells.** The MCF7 cell line is a breast cancer cell line obtained in 1970 from a female patient (85, 136). This cell line is characterized as differentiated mammary epithelium, which retained its ability to process estradiol via cytoplasmic estrogen receptors and the capability to form domes (25, 85, 136). Compared to MDA-MB-231 cells, MCF7 cells are less proliferative, less invasive and less metastatic (103). MCF7 cells express wild type p53 proteins.

**HL60 cells.** The HL60 cell line is derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. This cell line can be differentiated with treatment of phorbol myristic acid (PMA), butyrate, dimethylsulfoxide (DMSO) or retinoic acid. Once differentiated, these cells become phagocytic, which is extensively used as a substitute for phagocytes in molecular biological and biochemical research. This cell line is deficient of p53 tumor suppressor due to major deletions of the TP53 gene.

**H1299 cells.** The H1299 cell line is a human non-small cell lung carcinoma cell line derived from the metastatic lymph node (39). This cell line does not express p53 protein due to its homozygous partial deletion of the TP53 gene protein and do not express the tumor suppressor p53 protein (83) which partly account for their susceptibility to be more proliferative.

*Inhibitors*
Apigenin. Apigenin (4,5,7-trihydroxyflavone) (Figure 6A) is a nonmutagenic bioflavonoid found in leafy plants with properties such as anti-inflammatory, anti-oxidant and anti-carcinogenic (54). Studies have shown that apigenin can reverse cyclosporine-induced damage, stimulate adult neurogenesis and inhibit CYP2C9, an enzyme responsible for the metabolism of many pharmaceutical drugs in the body (111, 118, 127). During signal transduction, apigenin has been shown to inhibit PKC and MAPK activity, extracellular signal regulated kinase (ERK) and phosphorylation of EGFR tyrosine kinase (80, 111, 116, 134, 143). Dr. Cambronero’s laboratory has previously shown the inhibitory effect of apigenin on JAK3 kinase that led to reduced PLD2 activity and an associated lower cell invasion in MTLn3 cells (48).

5-Fluoro-2-indolyl des-chloroallopemide (FIPI). FIPI (Figure 6B) is a pharmacological PLD inhibitor that inhibits PLD1 and PLD2 activity with great potency both in vivo (0.5 nM IC\textsubscript{50}) and in vitro (25 nM IC\textsubscript{50}) (19). FIPI inhibits the catalytic activity of PLD and thus effectively blocks PA production (122). In addition, FIPI inhibits chemotaxis, F-actin cytoskeleton reorganization and cell spreading, which are regulated by PLD without interfering with the actin cytoskeleton, the cellular distribution of the proteins or access to its cofactor PIP\textsubscript{2}.

The PA sensor

For detection of endogenous PA using fluorescence microscopy, I used PA sensor. The PA sensor plasmid takes advantage of a 40-amino acid sequence found in Spo20 (Sporulation-specific protein 20) that binds to cell membrane phospholipids, particularly PA. Spo20 is a yeast protein required to the fusion of exocytic vesicles with the plasma membrane during yeast sporulation through its interactions with the SNARE complex (94-96). Spo has an inhibitory region that sequesters the protein in the nucleus (94, 133) and a positive regulatory region that
binds to phospholipids (including PA) in the cell membrane. This region contains an amphipathic helix with hydrophobic and positive charged faces. The initial report of a plasmid construction of the PA sensor in yeast, with the coding sequence of the PA binding domain (PABD) from Spo20p amino acids 51–91 was in Ref 142 (94). Cloning of the PABD in pEGFPC1 vector (Clontech) leading to pEGFP-Spo20PABD-wt for use in microscopy of mammalian cells and generation of the pEGFP-Spo20PABD-L67P mutant was performed in (146), where the authors show that PA is not present in cell membrane in resting cells but it becomes visible upon cell stimulation (PA is present in the nucleus of resting cells but that could be non-specific, no-PA related). Another use in mammalian cells with PLD inhibitor was reported in (122) and the first use in leukocytes and also R.E.D. analysis is in (37) indicating that the construct is cut by

![Diagram of Apigenin and FIPI](image.png)
ApaLRI at the pUC origin (in addition, Age and DraII are one cut, Pst is a two-cut, and NCoI is a four-cut restriction enzyme).
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

The MDA-MB-231 human breast cancer cell line is highly proliferative and metastatic, and also bears high PLD2 enzymatic activity (26) for reasons not well understood. I hypothesized that PLD2 plays an essential role in maintaining the high levels of proliferation that I observed in MDA-MB-231 cells. I specifically hypothesized that through enhanced activities of Fes and JAK3 tyrosine kinases, the PLD2 activity is enhanced.

To demonstrate this hypothesis, I developed a series of experiments with two Specific Aims:

AIM 1: A novel pathway governing cell proliferation exists between Fes, JAK3, and PLD2, and is heightened in cancer cells. In this part, I would like to find out the cellular events that lead to abnormally upregulated PLD2 activity in MDA-MB-231 breast cancer cells. I hypothesized that the lipase activity in MDA-MB-231 cells is related to high tyrosine kinase
activity known to be elevated in cancer cells, and I set out to compare these transformed cells with untransformed cells. To demonstrate this, I used the MDA-MB-231 cells as the highly proliferative, transformed cell model and, and MCF10A as a model of untransformed, normal cells and studied the enzymatic and protein expression levels of the following proteins: JAK3, Fes and PLD2. I used site-directed mutagenesis to make JAK3, Fes and PLD2 mutants that facilitate my investigation of their interactions. I also measured the intracellular levels of the phospholipids PA and its interaction with JAK3 and Fes kinases in both cell lines. Finally, I conducted experiments to investigate the differential effects of JAK3, Fes and PLD2 activities on MDA-MB-231 cells compared to MCF10A cells.

**AIM 2: Serum deprivation confers a survival mechanism operating through a pathway involving EGFR/JAK3/PLD2 on the MDA-MB-231 cell line.** As it is known that PLD2 can contribute to increased cell transformation upon epidermal growth factor (EGF) stimulation of cells and that MDA-MB-231 cells bear elevated PLD activity, I used these cells to study how the the highly invasive phenotype are regulated and if this regulation is PLD2-dependent under conditions of serum deprivation. The starvation serum contains Dulbecco’s Modified Eagle Media (DMEM) or Iscove’s Modified Eagle Media (IMEM) depending on the cells types, together with 0.5% bovine serum albumin (BSA). Application of starvation serum mimics the environments a cancer cell might encounter at the center of a tumor where there is insufficient nutrient. I also examined other cell types including COS-7, MCF7, H1299 and differentiated HL60 (dHL60). Further, I used the small molecule inhibitor apigenin to test if it could counteract the highly invasive phenotype of these breast cancer cells with a future potential therapeutic benefit.
V. MATERIALS AND METHODS

Materials

Dulbecco’s Modified Eagle Media (DMEM) and Iscove’s Modified Eagle Media (IMEM) were from Mediatech (Manassas, VA); Mammary Epithelial Cell Growth media was from Cell Applications, Inc. (San Diego, CA); Lipofectamine and Plus reagent were from Invitrogen (Carlsbad, CA); siQuest transfection reagent was from Mirus (Madison, WI); matrigels were from BD Biosciences (San Jose, CA); hematoxylin was from Ricca Chemical Co. (Arlington, TX); apigenin and FIPI were from Sigma (St. Louis, MO); CAY10594 was from Cayman Chemical (Ann Arbor, MI); protein G agarose beads, mouse anti-PLD2 IgG antibody (SKB2), purified recombinant human JAK3, purified recombinant human Fes and the Fes substrate peptide (poly(Glu4-Tyr) biotin-conjugated) were from Millipore (Billerica, MA); JAK3tide synthetic peptide substrate was from Anaspec (Fremont, CA); [³H]-butanol was from American Radiolabeled Chemicals (St. Louis, MO); [³²P]-γATP was from Perkin-Elmer
(Waltham, MA); ECL reagent was from GE Healthcare (Piscataway, NJ); rabbit anti-JAK3 and rabbit anti-Fes IgG antibodies were from Cell Signaling (Danvers, MA); rabbit anti-PLD2 (H-133) IgG antibody and donkey anti-rabbit TRITC IgG antibody were from Santa Cruz (Santa Cruz, CA); and si-Neg, siFES and siJAK3 were from Applied Biosystems (Foster City, CA).

Methods

Cells and cell culture. MDA-MB-231, COS-7 and HL-60 cells were obtained from ATCC. MCF-7, H1299 and MCF10A cells were a gift from Dr. Steven Berberich (Wright State University) and AML-3D10 cells were a gift from Dr. Michael Baumann (Wright State University). MCF-7, MDA-MB-231, H1299 and COS-7 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), while MCF10A cells were cultured in Mammary Epithelial Cell Growth with 0.4% (v/v) bovine pituitary extract, 10^{-7}% (w/v) epidermial growth factor, 5x10^{-4}% (w/v) insulin, and 5x10^{-5}% (w/v) hydrocortisone. Promyelocytic leukemic HL-60 cells and AML-3D10 cells were grown at 37 °C in a 5% CO2 incubator in Iscove’s Modified Eagle’s Media (IMEM) + 20% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine. Cell density was maintained between 0.1 and 1.0x10^6/ml. The plasmids used in these experiments were as follows: pcDNA3.1-mycPLD2-WT, pcDNA3.1-mycPLD2-Y169F, pcDNA3.1-mycPLD2-Y179F, pcDNA3.1-mycPLD2-Y415F, pCMV6-XL4-Fes-WT (Origene, Rockville, MD), pME1S-JAK3-WT (a kind gift from Dr. John O’Shea, NIH) and pEGFP-Spo20-PABD-WT (a kind gift from Dr. Michael Frohman, SUNY). The following mutant plasmids were prepared by site-directed mutagenesis by Mutagenex (Hillsborough, NJ): pCMV6-XL4-Fes-K590E (kinase-dead) and pCMV6-XL4-Fes-E469K/E472K (SH2 deletion.
mutant); pME1S-JAK3-K855A (kinase-dead) and pME1S-JAK3-R402K/R403K (SH2 deletion mutant).

**Cell transfection and gene silencing.** Transfections were performed using ~2 µg of plasmid DNA, 5 µl Lipofectamine (Invitrogen, Carlsbad, CA) and 5 µl Plus reagent (Invitrogen) in OptiMEM medium (Invitrogen) in sterile glass test tubes, per the manufacturer’s protocol. MDA-MB-231 and MCF10A cells were transfected for 3 h and were washed and refed with prewarmed complete medium. After 36 h, cells were harvested for their respective experiment.

Fes and JAK3 expression in cells was downregulated using small interfering RNA (siRNA) in combination with the siQuest transfection reagent (6 µl). Then up to 300 nM siRNA was used in the appropriate complete cell culture growth media and applied to the cells. Double-stranded RNA (dsRNA) was from Applied Biosystems (Foster City, CA) as “select validated.” For JAK3, I used a siRNA that targeted exon 19 [locus s7653; sense, 5’-GUAUCGUUGUGUCAGCUAUdT(TT)-3’]. For Fes, we used siRNAs that targeted exons 16 and 18 [locus s5113; sense, 5’-CCUCAGCAAUCAGCAGACAd(TT)-3’]. A negative control for siRNA was also from Applied Biosystems. Neg-siRNA2 is a 19-bp scrambled sequence with 3’-dT overhangs (sequence not disclosed by manufacturer). The cells were allowed to incubate with the siRNA in complete medium (minus antibiotics) for 3-4 days before being harvested.

**Detection of Intracellular PA Using a GFP-based Sensor.** The yeast (Spo20p) soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) PA binding domain (wtPABD) was cloned in a pEGFPC1 vector (Clontech) and denoted “pEGFP-Spo20PABD-WT”. The origin of the coding sequence of Spo20p amino acids 51–91 was reported in Nakanishi et al., who amplified it by PCR using BBO5 and PDO2 as primers and pRS306-
SPO20pr-SPO20 as a template and introduced XhoI and XbaI sites. This construct is cut by ApaLRI at the pUC origin (in addition, AgeI and DraI are one-cut, PstI is a two-cut, and NcoI is a four-cut restriction enzyme). The wild type PA binding sensor has been characterized. The intensity of green light (550nm) observed in the fluorescent microscopy is correlated to the amount of PA present in the cell, after cells are stimulated by agonist EGF. PA sensor-loaded cells can also display differences in PA subcellular localization.

MCF10A or MDA-MB-231 cells were transfected with 1.5 µg wild type PA sensor and/or a combination of 1.5 µg Fes-WT and/or 1.5 µg PLD2-WT and were grown on glass coverslips inside 6-well tissue culture plates. Two days post-transfection, fresh media containing no serum was added to the dishes, and cells were incubated with exogenous PA. After 15 min of stimulation, the medium was aspirated and cells were washed three times with phosphate-buffered saline to remove any traces of extracellular PA. Cells were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS), blocked in 10% fetal calf serum (FCS) in 0.1% Triton X-100 in PBS, (if relevant, probed with either rabbit anti-Fes or rabbit anti-PLD2 (H-133) IgG antibodies and then donkey anti-rabbit TRITC IgG antibody), mounted onto glass microscope slides using VectaShield mounting media and imaged using the FITC channel (DAPI was also included to visualize the nucleus). Cells were examined on a Nikon Eclipse 50i fluorescence microscope and photographed (SPOT camera; Diagnostic Instruments) by consecutive exposure using green, red and blue filters with a 100x (numerical aperture 0.9) OIL objective.

Cell invasion assays. Cells were serum-starved for 2 hours and resuspended at a concentration 1.5 x 10^6 cells/ml in chemotaxis buffer (DMEM + 0.5 % bovine serum albumin for MDA-MB-231, H1299 and MCF-7 cells or HMEM + 0.5 % bovine serum albumin for MTLn3 cells). Two
hundred µl cells were applied to the upper chambers of 8 µm PET Matrigels (24-well format) with a 6.5 mm diameter membrane (Figure 7). Final concentration of chemoattractant used was 0 or 3 nM EGF in 500 µl of chemotaxis buffer placed in the lower wells of 24-well plates. Cell invasion assays were incubated for 6 hr (MDA-MB-231 and H1299) or overnight (MTLn3 and MCF-7) at 37 °C in a humidified 5 % CO2 cell culture incubator. Cells were scraped from the Matrigel insert and were then stained for 1 hr in hematoxylin at room temp to visualize cells that invaded the Matrigel. The number of cells that migrated through the Matrigel to the bottom surface of the insert was calculated by counting 3 fields of cells using a 20x objective and a 10x ocular.

Figure 7. Schematic representation of Matrigel assay for cell invasion analysis.
Cell proliferation assay. Cells were plated into 24-well plates prior to use. Duplicate wells were untreated or treated with 300 nM siRNA for 0, 12, 24, 36, 38, 60 or 72 hr or were untransfected or transfected with 1.5 µg of various PLD2, Fes or JAK3 plasmid constructs for 60 hr. After incubation, cells were washed 2x with PBS and trypsinized and final volume brought up to 1 ml/well using complete DMEM containing 10% FCS. Fifty µl of trypan blue was added to each cell sample and viable cells counted/ml cells.

Co-immunoprecipitation and Western-Blot Analyses. After transfection, cells were harvested and lysed with Special lysis buffer (5 mM HEPES, pH 7.8, 100 µM Sodium orthovanadate and 0.1% Triton X-100). The lysates were sonicated and treated with 1 µl monoclonal antibody for the respective protein and 20 µl Protein G agarose beads and incubated at 4°C for 4 h. After incubation, the immunoprecipitates were washed with LiCl wash buffer (2.1% LiCl, 1.6% Tris-HCl, pH 7.4) and NaCl wash buffer (0.6% NaCl, 0.16% Tris-HCl, 0.03% EDTA, pH 7.4), respectively, and sedimented at 12,000 × g for 1 min. Using different types of salts (LiCl and NaCl here) removes effectively non-specific protein interaction with the agarose beads. Lithium
is also soluble in buffers containing high amounts of SDS and is hence used as a component of wash buffer. The resulting pellets were then analyzed using SDS-PAGE and Western-blot (W.B.) analyses.

For Western-blot analyses with or without immunoprecipitation, ~500 or 100 µg cell lysates, respectively, were separated using SDS-PAGE and then transferred onto Immobilon-P (PVDF) membrane for 1 hr at 400 mA. PVDF membranes were blocked in 5% bovine serum albumin (BSA), 0.5% Tween-20 in Tris-buffered saline (TBS-T) for 1 hr at room temp, probed with primary IgG antibodies overnight at 4 °C and then probed with secondary IgG HRP-conjugated antibodies for 1 hr at room temp. PVDF membranes were then exposed to ECL reagents and western-blot reactions were detected using autoradiography film.

**PLD activity assay.** Cell lysates were processed for PLD activity in PC8 (1,2-dioctanoyl-sn-glycero-3-phosphocholine) liposomes and [³H]n-butanol beginning with the addition of the following reagents (final concentrations): 3.5 mM PC8 phospholipid, 1 µM PIP₂, 45 mM HEPES (pH 7.8), and 1.0 µCi [³H]n-butanol in a liposome form, as indicated in ref. (31) to accomplish the transphosphatidylation reaction of PLD. Samples were incubated for 20 min at 30 °C with continuous shaking. As previously stated in our lab (4) synthesis of phosphotidyl butanol (pBut) is linear in the first 20 min and only begin to reach plateau at 30 min. Addition of 0.3 ml ice-cold chloroform/methanol (1:2) stopped the reactions. Lipids were then isolated and resolved by thin layer chromatography. The amount of [³H]-PBut that co-migrated with PBut standards (Rf=0.45-0.50) was measured by scintillation spectrometry.

**JAK3 and EGFR Kinase Assay.** Samples containing 2 x 10⁶ cells were either mock-treated or treated with 3 nM EGF for an 14 min in a 37 °C water bath with vigorous shaking. After
stimulation, cells were sedimeted, washed and finally lysed via sonication in 20 µl SLB containing protease inhibitors. Lysates were incubated in the presence of the following final concentration of each: 4 mM MOPS, pH 7.0, 15 mM MgCl₂, 1 mM EGTA, 0.2 mM Na Orthovanadate, 0.2 mM DTT, 1 µCi [³²Pγ]-ATP, 100 µM cold ATP and 42 µM JAK3tide substrate to yield a 40 µl total kinase reaction volume. Reactions were incubated at 30 °C for 20 min (tested in our lab that the kinase reaction does not reach plateau at 20-min time span) and stopped by spotting 20 µl reactions onto 2 x 2.5 cm² pieces of P81 Whatman filter paper for duplicate determinations. After filter papers were dry, each was washed in cold running water for 5 min total. Filters were dried and individual filters placed into scintillation vials containing Scintiverse II (Fisher) liquid scintillation cocktail. All samples were counted in a Beckman LS 6000TA liquid scintillation counter using the [³²P] protocol for 1 min each. Results were quantified as DPMs and expressed in terms of percent over control.

**FES Kinase Assay.** In the *in vitro* kinase assay, the phosphoacceptor peptide substrate was the FES substrate peptide: Poly (Glu₄-Tyr) biotin conjugated (Millipore) in freshly-prepared kinase buffer (8 mM MOPS, pH 7.2, 9 mM MgAc, 30 µM Na₂VO₃, 5 mM p-nitrophenyl phosphate, 1 mM EDTA, 2 µM cAMP-dependent kinase inhibitor, 0.420 mCi [γ³²P]ATP (7 nM), and 100 µM unlabeled ATP). To initiate the phosphotransferase reaction, aliquots (20 µl) of kinase buffer containing the *Fes* substrate peptide were mixed 1:2 (v/v) with the cell lysates (~20 µg protein). The reaction was carried out at 37 °C for 10 min (tested in our lab so the kinase reaction does not reach plateau at 10 min time span) in a tube rotisserie and terminated by adding 5 µl of 3% phosphoric acid, and blotting 30 µl of the reaction mixture onto SAM-2 biotin capture membranes (Promega). Membrane squares were extensively washed with methanol, water, dried,
and counted for radioactivity. Positive controls used recombinant fully-active FES (Millipore). Negative controls were run in parallel with no FES substrate peptide.

**PIP\(_2\) and PA liposome preparation.** The PA utilized in this work was a cell membrane soluble form, named “DOPA” (1,2-dioleoyl-sn-glycero-3-phosphate; Avanti Polar Lipids, Alabaster, AL) and was prepared by resuspending 1 mg DOPA in 1.4 ml liposome buffer (1× PBS plus 0.5% bovine serum albumin [BSA], pH 7.2). The solution was diluted in low bicarbonate DMEM plus 10% fetal calf serum (FCS) to achieve an intermediate stock of 1 mM and then sonicated at medium strength for 20 bursts of 5 s each while on ice. Intermediate dilutions were made in Hanks’ balanced buffer plus 0.5% BSA, pH 7.35, and applied to the cells. PIP\(_2\) was prepared by drying under a N\(_2\) stream to evaporate the CHCl\(_3\) and resuspended in liposome buffer, following the protocol as indicated above for PA.

**Statistical Analysis.** Data are presented as mean ± SEM. The difference between means was assessed by the Single Factor Analysis of Variance (ANOVA) test. Probability of p<0.05 indicated a significant difference.
VI. RESULTS

AIM 1: A novel pathway governing cell proliferation exists between Fes, JAK3, and PLD2, and is heightened in cancer cells.

A. A differential biochemical activity of Fes, JAK3 and PLD2 in non-transformed versus transformed cells

To begin the research project that led to this thesis, I measured the endogenous activity of JAK3, Fes and PLD2 in normal (MCF10A epithelial cells) and cancer cells (MDA-MB-231). Figure 8A,C,E shows that the MDA-MB-231 cancer cells possess greater endogenous JAK3, Fes and PLD activities when compared to the non-transformed MCF10A cells. I also investigated the activities of JAK3, Fes and PLD2 in a range of cell types (results not shown) and found a similar pattern for catalytic activities, i.e. the kinase and lipase activities are higher in transformed cells (MCF10A breast cancer cells, eosinophilic AML14.3D10 cells, leukemic HL60 cells and ) than in untransformed cells (kidney epithelial fibroblast COS7 cells).
Figure 8B,D,F shows the endogenous expression of each of the proteins in untransformed MCF10A and MDA-MB-231 cancer cells. PLD2 level is significantly higher in the cancer cells than in MCF10A cells (Figure 8F). The protein levels of JAK3 in MDA-MB-231 cells are higher but to a lesser extent than in MCF10A cells (Figure 8B). I also found that the Fes protein level is approximately similar in MCF10A cells to those in MDA-MB-231 cells (Figure 8D). Therefore, the reason of significant higher JAK3 and Fes kinase activities in MDA-MB-231 cells is highly unlikely to be a result of higher protein expression.

**Figure 8. Endogenous JAK3, FES, and PLD2 activity levels in untransformed and transformed cells.** JAK3 (A), Fes (C) and PLD2 (E) activities were measured in MCF10A and MDA-MB-231 cell lysates starting from similar number of cells per sample and then adjusting
for similar protein levels per sample. All 3 activities are elevated in MDA-MB-231 cancer cells compared to non-transformed MCF10A cells. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbol * denotes statistically significant (p<0.05) differences (increases) between samples and controls. Western blot analysis of endogenous JAK3 (B), Fes (D) and PLD2 (F) proteins (~100 µg/lane) in non-transformed MCF10A and transformed MDA-MB-231 cancer cell lines with actin expression included as protein loading controls.

**B. JAK3, Fes and PLD2 mutants are designed and characterized.**

Mutant forms of JAK3, Fes and PLD2 were generated (Figure 9A) to study to what extent tyrosine kinases JAK3 and Fes may regulate PLD2. The catalytic sites were targeted on each kinase to generate kinase dead mutants JAK3-K855E (JAK3-KD). SH2 binding deficient forms of JAK3 (JAK3-SH2-L-i) and Fes (Fes-SH2-i) were also generated, which target residues R402/R403 in the SH2-like domain of JAK3 and residues E469/E472 in the SH2 domain of Fes. Regarding PLD2, Two tyrosine residues Y169 and Y179 situated within the YxN motif that is recognized by SH2-bearing proteins and Y415 which is essential for JAK3 binding (47) were targeted to generate SH2-binding deficient mutants PLD-Y169F and PLD-Y179F and JAK3-binding deficient mutant PLD-Y415F. Each mutant is generated by point mutation/mutations using site-directed mutagenesis.

The left panels of Figure 9B-D demonstrate the expression levels of the engineered plasmid constructs in Western blot analysis, all of which showed similar expression. It is also shown here the impact of mutations on enzymatic activities (Figure 9B-D, right panels). Lysine
to Glutamic Acid mutations for both JAK3 (K855E) and Fes (K590E) in their respective kinase domain rendered the respective kinases to low basal level. Double-point mutations in the SH2-like and SH2 domains of JAK3 and Fes, respectively, do not affect JAK3 activity, but affect Fes activity to some extent as the SH2 domain has been implicated for optimal Fes activity (35). It is still viable for its study since the Fes-SH2-i mutant maintained most Fes activity.
Figure 9. Analysis of wild type and mutant JAK3, Fes and PLD2 proteins. (A) The sites of mutagenesis in JAK3 are indicated, including R402 and R403, which are important for ligand binding and K855 which is important for kinase activity. Fes has a Tyr kinase domain and a Src homology 2 (SH2) domain that bind to ligands and enhance kinase activity. The F-BAR and FX domains are involved in phospholipid binding, membrane localization and kinase activity. The sites of mutagenesis in Fes are indicated including E469 and E472 important for ligand binding and K590 essential for kinase activity. The sites of mutagenesis in PLD2 are indicated including Y169 and Y179 which are important for binding of SH2-containing proteins and Y415 which is important for JAK3 binding. (B-D) Western blot analyses of expression of recombinant wild-type and mutant JAK3, Fes, and PLD2 proteins (~100 µg/lane), respectively. These experiments were conducted to function as controls to demonstrate comparable expression of each protein and their corresponding mutants in subsequent experiments that used the recombinant proteins. JAK3-K855E and Fes-K590E are both kinase-dead mutants. JAK3-R402K/R403K and Fes-E469K/E472K are JAK3 SH2-like (JAK3-SHLi) and Fes SH2 (Fes-SH2i) domain mutants, respectively. PLD2-Y169F and -Y179F are SH2 binding domain mutants (PX domain). PLD2-
Y415F is a tyrosine mutant that prevents phosphorylation and activation through JAK3. Kinase and lipase activities in vitro of cell lysates that overexpress the three proteins of interest used in this study (JAK3, Fes and PLD2). Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.

**D. JAK3 kinase regulates PLD2 in both transformed and transformed cells**

Our lab has previously demonstrated phosphorylation of certain tyrosine residues within PLD2 upon stimulation of cells (49). To examine whether this phosphorylation occurred due to JAK3 activity, I devised a series of experiments. Figure 10A left panel demonstrates the impact of silencing JAK3 in MDA-MB-231 or MCF10A cells. Silencing JAK3 decreased lipase activity in MDA-MB-231 cells, but increased PLD activity in MCF10A cells, suggesting a positive role of JAK3 on PLD2 activity in the cancer cells but a negative one in MCF10A cell. The right panel is a control that shows effective silencing of JAK3 using siRNA.

Figure 10B demonstrates JAK3-WT or JAK3 mutant plasmid constructs expressed in MCF10A or MDA-MB-231 cells and the resulting effect of the expression of these constructs on PLD2 activity in vivo. JAK3-WT decreased lipase activity to a moderate degree in MCF10A cells but had a significant positive effect in cancer cells. The kinase dead mutant JAK3-KD reduced PLD2 activity significantly suggesting the possibility of JAK3 phosphorylation and activation of PLD2. Even though we are aware of the possibility that more rapidly dividing MDA-MB-231 cells take up more plasmid so higher percentage of cells are transfected than MCF10A cells, we have seen that protein expression in both cell types are approximately similar.
Figure 10C demonstrates the effect that increasing amounts of DNA constructs for JAK3 or JAK3 and PLD2 have on PLD2 activity \textit{in vivo} in MDA-MB-231 cells. The results are consistent with the finding that JAK3 acts as a positive regulator of PLD2 activity in the cancer cells. Recombinant purified JAK3 was also utilized and induced a significant increase in PLD2 activity after incubation with either MDA-MB-231 cells or recombinant purified PLD2 proteins.
Figure 10. Effect of JAK3 on PLD2 activity in non-transformed versus transformed cells.

(A) Effect of silencing JAK3 on endogenous PLD activity in MCF10A and MDA-MB-231 cells (300 nM siRNA per transfection; 4 day-silencing). MCF10A cells saw a slight increase in PLD2 activity with siJAK3, while MDA-MB-231 cells saw a significant decrease in siJAK3. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (Figure 10 legend continued)

(increases or decreases, respectively) between samples and controls. (Inset) Evidence of JAK3 silencing using short interfering RNA (300 nM siRNA per transfection; 4 day-silencing) in MCF10A and MDA-MB-231 cells. Western blots were performed to determine that JAK3 was effectively silenced (~100 µg/lane). Actin is shown as an equal loading control. (B) JAK3-WT or JAK3-KD were each overexpressed in MCF10A and MDA-MB-231 cells, and PLD activity was
measured from the resulting cell lysates. Overexpression of JAK3-WT and Fes-WT modestly increased PLD activity in MCF10A cells, while there was a much more significant increase in PLD activity in MDA-MB-231 cells. (C) Increasing JAK3-WT overexpression has a positive effect on both endogenous and overexpressed PLD activities in MDA-MB-231 cancer cells in vivo and in vitro, which can be reversed in the presence of the tyrosine kinase inhibitor, apigenin.

The isoflavone, apigenin, was also used as a JAK3 inhibitor which abrogated the positive effect of JAK3 on PLD2 activity.

E. Fes modulate PLD2 activity differently in transformed cells compared to untransformed cells due to differential interaction sites on PLD2

As it was previously known that Fes possesses an SH2 domain (Figure 4), I reasoned that this SH2 could interact with PLD2 at the two tyrosine residues located within the PX domain of PLD2. Using the Fes-SH-i mutant, together with the Fes-KD mutant and PLD2-YF mutants, I examined the association between Fes and PLD2. In Figure 11A left panel, PLD2 activity is assayed when Fes is silenced in both cell types. Fes depletion has a negative impact on PLD2 activity in both the transformed and untransformed cells. Such negative impact is more significant in MDA-MB-231 cells than in MCF10A cells. Fes is therefore a positive regulator in both cell types but has a greater positive effect on the cancer cells. The right panel is a control that shows effective silencing of Fes using siRNA.

In Figure 11B, I demonstrate that PLD2 activity in MDA-MB-231 cells is affected by deficiency of the SH2 domain and loss of kinase activity in Fes. On the other hand, in MCF10A cells, loss of Fes kinase activity reduced PLD2 activity to basal level while overexpression of
SH2-inactive Fes mutant did not affect PLD2 activity. MCF10A cells showed a marked decrease in PLD2 activity upon transfection with Fes-WT. The kinase-inactive Fes (K590E) displayed a decrease in lipase activity in both cell lines.

I next examined how lipase activity was affected by mutations in PLD2 in context with wild-type Fes (Figure 11C). With the presence of overexpressed Fes, both PLD2-Y169F and PLD2-Y179F rendered PLD2 activity to basal level, while overexpression of PLD2-Y415F
Figure 11. Effect of Fes on PLD2 activity in non-transformed versus transformed cells. (A)
Effect of silencing Fes on endogenous PLD activity in MCF10A and MDA-MB-231 cells (300 nM siRNA per transfection; 4 day-silencing). MCF10A cells saw a slight increase in PLD2 activity with siFes, while MDA-MB-231 cells saw a very significant decrease in siFes. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbol # denotes statistically significant (p<0.05) decreases between samples and controls. (Inset) Evidence of Fes silencing using short interfering RNA (300 nM siRNA per transfection; 4 day-silencing) in MCF10A and MDA-MB-231 cells. Western blots were performed to determine that Fes was effectively silenced (~100 µg/lane). Actin is shown as an equal loading control. (B) PLD2 activity in MCF10A and MDA-MB-231 cells over expressing Fes-WT, Fes-KD, or Fes-SH2 deficient plasmids (2 µg DNA per transfection; 2 day-transfection). MDA-MB-231 cells show a significant increase with Fes-WT transfection, an effect which was abrogated with SH2 mutant expression. (C) PLD2 activity in MCF10A or MDA-MB-231 cells transfected with PLD2-WT, Fes-WT or both or co-transfections of PLD2-WT or its mutants, PLD2-Y169F, PLD2-Y179F or PLD2-Y415F (2 µg DNA per transfection; 2-day transfection) with Fes-WT. The decrease in PLD activity of MDA-MB-231 cells that overexpressed both Fes-WT and the PLD2 mutants Y169F and Y179F demonstrate a key role in regulating the Fes kinase activity through these two residues on PLD2 in transformed cells. For MDA-MB-231 cells, a decrease occurs when Fes-WT and PLD2-Y415F mutants were both overexpressed suggesting the residue 415 on PLD2 is important for PLD2 interaction with Fes. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls. (D) PLD2 and Fes form a protein:protein complex. Panels demonstrate co-immunoprecipitation of overexpressed PLD2 and Fes proteins in both
MCF10A and MDA-MB-231 cells. Immunoprecipitations were performed with either rabbit anti-myc (PLD2) or rabbit anti-Fes IgG antibodies bound to Protein G agarose beads, and after SDS-PAGE, the resulting western blots were probed with either rabbit anti-myc or rabbit anti-Fes IgG antibodies, respectively. Anti-myc antibodies were used for immunoprecipitations, and the anti-Fes antibodies were used to probe the western blots (top panels). Fes was detected at the native molecular weight of ~95 kDa (second panels from top). Equal protein loading controls were detected using rabbit α-actin antibody (second panels from bottom). Negative controls using IgG antibody for co-immunoprecipitations are included in the bottom panels for each cell line. Binding of Fes-SH2 mutant to PLD2 is decreased in MDA-MB-231 cancer cells compared to MCF10A cells. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate.
plasmid did not affect PLD2 activity. This in combination with the finding in Figure 4B that the Fes-SH2-i mutant knocked down PLD2 activity to basal level, suggesting the interaction between Fes and PLD2 in MDA-MB-231 cells is through the SH2 domain in Fes and the SH2-binding motif (where Y169 and Y179 are located) of PLD2. In contrast, when Fes was co-transfected, deficient in SH2-bearing protein binding in PLD2 (PLD2-Y169F and PLD2-Y179F) did not affect the lipase activity while mutation at Y415 residue affected the lipase activity. Taken together the finding that Fes-SH2-i mutant does not affect PLD2 activity in MCF10A cells (Figure 11B), the interaction between PLD2 and Fes is mediated by PLD2-Y415 and site on Fes other than the SH2 domain.

Figure 11D shows an anti-PLD2 immunoprecipitation followed by Western blotting with anti-Fes antibodies. The presence of a band at the corresponding molecular weight marker for Fes denotes the presence of Fes:PLD2 binding in both MCF10A and MDA-MB-231 cells. Furthermore, when I utilized the SH2-inactive Fes-SH2-i mutant along with endogenous PLD2, Fes expression was reduced in MDA-MB-231 cells. This indicates that Fes binds to PLD2 SH2-binding motif through Fes’s SH2 domain in the cancer cells. In contrast, in MCF10A cells, a slightly darker band shows a stronger interaction between Fes-SH2-i and endogenous PLD2 illustrating that the SH2 domain of Fes is not important in PLD2 binding in untransformed cells. The reason of the presence of stronger binding between Fes-SH2-i mutant and PLD2 will be discussion in the later section.
D. Fes is differentially modulated by phospholipids in untransformed and transformed cells

As PLD2’s major enzymatic activity generates PA, I wanted to examine if there is any interaction between PA and the two kinases under investigation. For this, I used a PA sensor which is a plasmid (Figure 12A) containing a 40-amino acid sequence found in yeast Spo20 (Sporulation-specific protein 20) that binds to cells membrane phospholipids, particularly PA. Cloning of the PA binding domain (PABD) in pEGFPC1 vector leads to pEGFP-Spo20PABD-wt for use in microscopy of mammalian cells. Therefore, the PA sensor can be transfected to detect PA levels in fixed cells. The intensity of GFP (in the FITC channel of a fluorescence microscope) correlates with the existing amount of PA.

In my study, I transfected MDA-MB-231 and MCF10A cells with PA only or PA in combination with Fes coupled with EGF stimulation (Figure 12A). The upper panels in Figure 12A show that there are more endogenous PA in MDA-MB-231 cells than in MCF10A cells. In addition, PA is nuclear in MCF10A cells while perinuclear in MDA-MB-231 cells. After EGF treatment, PA is translocated to cytoplasm in both cell types (Figure 12A lower panels). A significant increase in the endogenous PA level was seen upon Fes co-transfection in MDA-MB-231 cells and a moderate one in MCF10A cells (Figure 12B upper panels). Further increase of PA level is also seen with EGF treatment in both cell lines (Figure 12B lower panels). Again, both the nuclear PA in MCF10A cells and the perinuclear PA in MDA-MB-231 cells translocated to the cytoplasm where it is active after EGF stimulation.
Figure 12. Interplay between phosphatidic acid (PA) and Fes and JAK3 kinases. (A) MCF10A and MDA-MB-231 cells were plated onto glass coverslips and then transfected with 1 µg Fes plasmid in combination with a 1 µg of the PA sensor (EGFP-based) plasmid. Two-days
post-transfection cells were serum-starved for 2 hr and then stimulated with 3 nM EGF for 15 min. Cells were fixed onto coverslips with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS and then blocked with 0.1% Triton X-100 in PBS. Coverslips were probed with 1:200 Mouse anti-Fes IgG antibody and then Goat anti-mouse TRITC IgG antibody to visualize Fes staining. Green denotes the EGFP-tagged PA sensor. Blue denotes DAPI staining of the nucleus. Upper row, staining of the PA sensor (FITC) and nuclei (DAPI) only in cells in the absence of EGF. Lower row, staining of the PA sensor (FITC) and nuclei (DAPI) only in cells in the presence of EGF. (B) Upper row, staining of the PA sensor (FITC) and nuclei (DAPI) only in cells in the presence of Fes-WT (TRITC) in the absence of EGF. Lower row, staining of the PA sensor (FITC) and nuclei (DAPI) in the presence of EGF. (C) Effect of 100 nM lipids (PA, PIP$_2$ or PC) on recombinant JAK3 and Fes activities in vitro. (D) Differential effect of PA (DOPA, PA in a di-oleoyl or membrane-soluble form) on endogenous Fes activity of MDA-MB-231 and MCF10A cells. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.
I next investigated the effect of addition of the phospholipid PA (The product of PLD2 enzymatic activity) on Fes and JAK3 kinase activities from either cell lysates or from whole cells in the first part (Figure 12C). Cell lysates containing Fes or JAK3 proteins were incubated with PA and were then used to measure Fes and JAK3 activities. PIP2 is another phospholipid known to function as an intracellular messenger. PC was used as a control in these experiments. PA significantly activated Fes activity in the cell lysates. I also observed that PIP2 activates Fes, which is a novel finding. I have seen previously that PIP2 acts through 1-phosphatidylinositol-4-phosphate 5-kinase (PI5K; also activated by PA) and is also able to stimulate other proteins such as WASp (63). Despite the change in Fes activity, no change was detected in kinase activity of JAK3 in the presence of PA as well as PIP2. In Figure 12D, I examined Fes kinase activity in whole cells upon treatment with PA. I saw an increase in Fes activity in MDA-MB-231 cells, but found no significant effect on MCF10A cells.

**E. JAK3 and Fes regulate each other**

To examine the interaction between Fes and JAK3, I utilized siRNA for JAK3. In Figure 13A, I examined the effect of siJAK3 on Fes activity in a broad range of cell lines. All cells tested except COS-7 cells showed that JAK3 has a positive effect on Fes activity. Figure 13B demonstrates the effect of overexpressing Fes on JAK3 activity. In both MCF10A and MDA-MB-231 cells, JAK3 activity is up-regulated by Fes expression but at a greater extent in the latter. Figure 13C examines Fes activity following JAK3 overexpression. Fes activity was augmented in MDA-MB-231 cells but decreased in MCF10A cells upon JAK3 transfection. Taken together, the kinases showed influences on each other, with JAK3 having a positive impact on Fes in both cell types and Fes has a positive impact on MDA-MB-231 cells but a negative one on MCF10A cells.
Figure 13. Interaction between JAK3 and Fes kinases. (A) Fes activity in non-transformed MCF10A and COS-7) and transformed (MDA-MB-231, MCF-7, AML-3D10 and dHL-60) cells following silencing of JAK3. (B) Fes activity of MCF10A and MDA-MB-231 cells following transfection of Fes or JAK3 plasmid DNA (the Fes plasmid was uses as a positive control for Fes kinase activity). (C) JAK3 activity of MCF10A and MDA-MB-231 cells following transfection of Fes or JAK3 plasmid DNA (the JAK3 plasmid was uses as a positive control for JAK3 kinase activity). Panel B indicates an effect of JAK3 on Fes more prevalent than the converse, Fes on
(Figure 13 legend continued)

JAK3 (Panel C). The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate.
F. JAK3, Fes and PLD2 regulate cell proliferation

Finally, I examined the biological significance of the roles of JAK3, Fes and PLD2 in the transformed cells and untransformed cells. First I transfected cell with DNA constructs and 2 days after this, I performed cell proliferation assay. In Figure 14A, PLD2 accelerated cell proliferation in both transformed cells and untransformed cells. Fes overexpression has slight positive effect of cell proliferation while a highly significant one in MDA-MB-231 cells. Further, cell proliferation was slightly positively affected upon JAK3 overexpression in the untransformed cells while being highly accelerated in MDA-MB-231 cells. Although I saw that JAK3 inhibits PLD2, JAK3 here is able to stimulation cell growth since JAK3 has been implicated as a cell proliferation factor by interacting with other signaling molecules.

I also examined the change in cell proliferation when JAK3, Fes and PLD2 are silenced, individually or in combinations. As shown in Figure 14B, silencing PLD2 and Fes slowed cell growth in both cell lines while to the lesser extent in the untransformed cells. Depletion of JAK3 slightly increased cell growth in the MCF10A cells but showed a significant inhibition of cell growth in the cancer cells. When the three proteins are transfected together, proliferation is decreased in both cell lines but such negative impact is greater in the MDA-MB-231 cancer cells.

G. The existence of a new signaling pathway: JAK3 → Fes → PLD2 and mechanism of inter-regulation

Figure 15 left presents the putative series of events that could be taking place in transformed cells, cells that have a heightened JAK3→Fes→PLD2 pathway with respect to untransformed cells. This new pathway affords the cell several regulatory points that are up-regulated in the human breast cancer cell line MDA-MB-231. The reasons for elevated PLD2
Figure 14. The effect of PLD2, Fes or JAK3 overexpression or silencing on cell
proliferation in non-transformed versus transformed cells. (A) Cell proliferation of MCF10A or MDA-MB-231 cells in the presence of overexpressed PLD2, Fes or JAK3 60 hr post-transfection. (B) Cell proliferation of MCF10A cells at varying time points using Control siRNA, siJAK3, siFes+siPLD2 or siJAK3+siFes+siPLD2 (300 nM each). (C) Cell proliferation of MDA-MB-231 cells with similar conditions as Fig. 7A. Silencing all three proteins in transformed MDA-MB-231 cells (C) leads to a more significant decrease in cell proliferation when compared to the untransformed MCF10A cells (B). Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.
Figure 15. Model for the new PLD2-Fes-Jak3 pathway. In MCF10A cells, both the protein expression levels and the enzymatic activity of the three signaling molecules (PLD2, JAK and Fes) are maintained at a low level. A complex inter-regulation equilibrium composed of activations and inhibitions keep PLD and the synthesis of PA in balance and a basal level. In MDA-MB-231 cells, the situation is very different; both the protein expression levels and the activity of PLD2, JAK and Fes are up-regulated. The kinases activate PLD that is then synthesizing higher amounts of PA. Further, this PA feeds back positively on Fes that upon binding to the phospholipid is further activated.
activity in cancer cells appear to be multiple and can be thought out as follows: (1) EGF-R activation leads to PLD2 up-regulation and to a quick and robust PA generation; (2) parallel to this event, Fes autophosphorylates and also phosphorylates JAK3 which increases Fes activity; (3) PA generated by PLD2 upregulates Fes for its further activation and, in turn, Fes interact with PLD2 through the SH2 binding motif and stimulates it. (4) JAK3 activation contributes to higher PLD2 activity and it also interacts with other signaling molecules which lead to up-regulated abnormal cell proliferation in MDA-MB-231 cells.

In MCF10A untransformed cells (Figure 15 left), Fes has inhibitory effect on JAK3 so that JAK3 activity is at basal level. Fes does not interact with PLD2 by its SH2 domain but at sites that remains to be discovered. Since substrate binding to the SH2 domain is necessary to optimal Fes activity, Fes activity is low in these cells. JAK3 keeps PLD2 activity low by interacting with PLD2 through the PLD2-Y415 residue, while Fes also competitively binds to PLD2. Due to the lower activities of these two tyrosine kinases JAK3 and Fes, PLD2 activity is kept in balance that is less abundant in untransformed MCF10A cells.
AIM 2: Serum deprivation confers on the MDA-MB-231 cell line a survival mechanism operating through a pathway involving EGFR/JAK3/PLD2.

A. JAK3 is a major regulator of PLD2 and cell invasion

In the previous aim I have studied PLD2 with respect to cell proliferation. In this part, I investigated a possible role of PLD2 in cell invasion. I measured both PLD enzymatic activity and cell invasion in Matrigel. In Figure 16A and 16B I demonstrated that JAK3 was a critical regulator of PLD2 activity in both leukemic cell lines dHL-60 and 3D10 cells, extending our understanding of JAK3 regulation past the previously studied cell line. However, I discovered that JAK3 actually caused a decrease in PLD2 activity that was statistically significant in MDA-MB-231 cells (p<0.05 by t-test).

I silenced JAK3 in Figure 16C, and demonstrated the converse of what was shown in Figure 16A and 16B. Again, JAK3 appeared to have an inhibitory role in MDA-MB-231 cells in terms of lipase activity. Next I performed a Matrigel invasion analysis of MDA-MB-231 cells. Figure 16D demonstrates that invasion of cells was abrogated by JAK3 overexpression. Furthermore, invasion of cells was enhanced when silencing RNA targeting JAK3 was utilized. Taken together, these results suggest that PLD2 mediated cell invasion is inhibited by JAK3. It should be noted that these results (Figure 16) are the result of non-starved cells, wherein previous results (relevant to Aim 1) were done using serum starved cells. JAK3 seems to activate PLD2 upon serum starvation.

B. Serum deprivation induces JAK3 and PLD2 activity elevation

It is known that cancer cells are sensitized to stress. Upon oncogenic transformation, a cell activates its stress mechanism for protective purposes. In order to survive, transformed cells
Figure 16. PLD2 is inhibited by JAK3 in exponentially growing MDA-MB-231 cells. (A) Analysis of endogenous PLD activity in exponentially growing cancer cells. Leukemic cell lines HL-60 and eosinophilic Eo-3D10 were maintained in suspension, whereas breast cancer MDA-MB-231 were maintained in monolayer cultures. Cell lysates (~1 mg/ml protein concentration) were prepared and incubated with 1 µg of recombinant JAK3 (RecJAK3) for 20 min and then used for PLD activity measurement with PC8 in liposomes and [3H]-butanol as indicated in Material and Methods. (B) Analysis of transected PLD activity in exponentially growing cancer cells. Cells were transfected with myc-pcDNA3-PLD2-WT (2 µg DNA per condition) for two
days. After this, they were lysed and incubated with 1 µg of recombinant JAK3. (C) Effect of silencing JAK3 in cancer cells. Cells were transfected with either dsRNAs against JAK3 (si-JAK3) or siControl, at 250 nM, for four days, after which PLD activity was assayed in vitro. (D) Analysis of cell invasion. After transfection with either DNA (PLD2 construct) or RNA (silencing), cells were resuspended in RPMI-based chemotaxis buffer and 1x10^4 cells were placed on the upper or "insert" chambers of 6.5-mm, 8-µm pore diameter, and modified Transwell inserts layered with “Matrigel”. To begin cell invasion, 3 nM EGF was added to the bottom wells. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.
have to suppress stress signals that drive cells to senescence or move to locations with less stressful environment. In this part, I wanted to ascertain if stress conditions similar to those that a cancer cell might encounter (such as nutrient deprivation) could affect PLD activity. I speculated that the regulation of PLD2 regulation in MDA-MB-231 cells by JAK3 was due to differences in PLD2 expression and management of PLD2 activity by other effector molecules. It has previously been demonstrated that serum starvation is a factor which activates PLD2, which generates survival signals (148). In Figure 17A, I present data that indicates that JAK3 becomes a positive regulator of PLD2 mediated invasion after 16 hours. After two hours, JAK3 loses its inhibitory function against cell invasion.

In addition to an increase in cell invasion upon starvation, I also noted increases in PLD2 and JAK3 enzyme activities. In Figure 17B and 17C, graphs show the increase in PLD2 and JAK3 activities upon further starvation of cells. Apart from starvation, it appears that downward shifts in media pH also affect activation of the two proteins. Cells cultured in minimum nutrient media can be rescued by addition of 15% human serum.

C. The small molecule inhibitor Apigenin allowed dissecting out the inter-regulation between JAK3 and PLD2

To generate a better understanding of JAK3’s role in PLD2-mediated cell invasion in breast cancer cells, the flavonoids apigenin (4’,5,7-trihydroxyflavone) and Genistein and the quinazoline Janex-1 were used as inhibitors of JAK3. Figure 18A demonstrates that apigenin is the best inhibitor of JAK3 activity in vivo as compared to the other flavonoids used. Apigenin induces >60% inhibition at a concentration of 30 nM and >80% at a concentration of 100 nM. Based on the promising results that were seen with apigenin’s inhibition of JAK3, I next
Figure 17. JAK3 stimulates cell invasion after cell starvation. (A) Effect of increasing concentrations of a JAK3 transfection construct on cell invasion of non-starved or 2-hr or 16-hr...
starved MDA-MB-231 cancer cells. Two days after transfection with JAK3, cells were washed off the regular media and incubated for the indicated times in serum-free media. (B) Effect of serum deprivation on JAK3 kinase activity of MDA-MB-231 cancer cells. Cells were cultured in regular media (Control), in serum-free media, in minimum media (OptiMEM), in pH=6.5 media or in serum-free media for 16 hr and 15% human serum added back for 1 hr. 100% JAK3 activity represents 5,940 ± 50 cpm/mg protein. (C) Effect of serum deprivation on PLD lipase activity of MDA-MB-231 cancer cells, under the same experimental conditions as in panel (B). 100% PLD activity represents 2,327 ± 20 cpm/mg protein. Results in this figure are the means ± SEM from 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.
Figure 18. The JAK3 tyrosine kinase inhibitor Apigenin inhibits cell invasion. (A) Testing the potency of 3 tyrosine kinase inhibitors in JAK3 activity. Shown is the effect of increasing concentrations of apigenin, genistein and Janex-1 (each dissolved in 1 µl DMSO, final concentration) on MDA-MB-231 cells in vitro. Control cells received DMSO (1 ml/ml, final concentration) only. (B,C,D) Effect of increasing concentrations of apigenin on cell invasion in MDA-MB-231 (B), MTLn3 cancer cells (C), and H1299 cancer cells (D) in Matrigel using 3 nM EGF as chemoattractant for 16 hours. Control cells received no EGF (0 nM EGF). In the x-axis,
(Figure 18 legend continued)

for zero apigenin in both 0 nM EGF or 3 nM EGF, cells were incubated with DMSO alone (1 μl/ml, final concentration). Results in each panel are the means ± SEM from 3 independent experiments conducted in duplicate.
performed experiments to see if apigenin could abrogate cell invasion mediated by JAK3 in MDA-MB-231 cells and if these results could be extrapolated to other cancer cell lines.

Figures 18B-D demonstrates that apigenin has a robust inhibitory effect in the three cancer cell lines. To further investigate this I returned to the subject of this study, MDA-MB-231, and asked if cell invasion was inhibited depending on the time of exposure. Figure 19A,B indicates that apigenin (at 3 nM) inhibits responses to EGF at about nine hours. Thus, apigenin, at nanomolar concentrations, appears to be a powerful inhibitor of cancer cell invasion.

D. JAK3 is upstream PLD2 in signaling and positively modulates it

The next logical step was to examine whether apigenin had an effect on other tyrosine kinases other than JAK3. As cells were stimulated with EGF, I concentrated on the EGF receptor (EGFR). Figure 20A shows that apigenin robustly inhibits JAK3 but it also decreases EGFR activity, although at a lesser extent than it does JAK3. Dose-response curves indicate that apigenin inhibits the two kinases with IC$_{50}$ of around 1 nanomolar for JAK3 and twenty nanomolar for EGFR, with maximal inhibitions of ~80% and ~50%, respectively. Next, I measured PLD activity when cells were subjected to overexpression of tyrosine kinases EGFR and JAK3 constructs, and also the effect of both apigenin inhibition and JAK3 silencing (the latter as a more direct test of JAK3 than apigenin that can also inhibits EGFR, Figure 20A). Figure 20B and D demonstrate that: (a) JAK3 and EGFR overexpression enhances endogenous PLD2 activity within MDA-MB-231 cells; (b) PLD activity is at its highest when PLD2 is co-transfected along with JAK3 or with EGFR, and negated by dsRNA silencing; and (c) apigenin does not inhibit PLD2 directly, but rather it negates the increasing effect it receives from JAK3.
Figure 19. Apigenin inhibits both the rate and the extent of cell invasion. (A) Effect of cell migration in the presence of apigenin (open circles), EGF (filled squares), EGF with apigenin (open squares) and control (filled circles). MDA-MB-231 cells were pre-incubated with Apigenin and transferred to the insert of the Matrigel upper wells. Lower wells received, were appropriate, 3 nM EGF as chemoattractant. (B) In selected samples, treated as indicated in (A), cells that had invaded the Matrigel layer and migrated to the underside of the filter were processed. Shown are representative hematoxylin-stained Matrigel images with increasing concentration of apigenin as a function of time, visualized in an optical microscope at x20.
Figure 20. JAK3 effect on PLD2 is reversed by apigenin and by silencing JAK3. Even though apigenin does not inhibit PLD activity directly, it acts upon JAK3 that itself regulates PLD2. (A) Effect of increasing concentrations of apigenin on EGFR or JAK3 tyrosine kinase activities measured in vitro. (B,C,D) Effect of overexpression (B) or silencing (C,D) of JAK3 on PLD2 activity in MDA-MB-231. Shown in (D) is a Western blot of cell lysates form MDA-MB-231 silenced for 4 days with dsRNA against JAK3, with actin levels included, for protein loading controls. Results in this figure are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.
(but slightly less form EGFR). All this indicates that JAK3 is above PLD2 in a cell-signaling pathway with JAK3 modulating (positively) the phospholipase. As known, EGFR is a membrane-receptor tyrosine kinase that sits above JAK3, indicating that the system at hand is operating as an EGFR/JAK3/PLD2 pathway.

**E. Cell invasion is enhanced by PLD2 and JAK3 but abrogated by Apigenin and FIPI treatment**

In addition to tyrosine kinases, I examined serine/threonine kinases in evaluation inhibition through apigenin. I utilized S6K and mTOR plasmids that our laboratory possessed as two models of serine/threonine kinases. Figure 21A demonstrates that all four kinases alongside PLD2 are capable of enhancing cell invasion, with the following order: PLD2 > EGFR > JAK3 > S6K ~ mTOR. Despite apigenin’s lack of direct inhibition of PLD2, it still is capable of inhibiting cell migration through kinases. As expected, serine/threonine kinases like mTOR or S6K were not affected by apigenin and theses were taken as important negative controls. Interestingly, mTOR and S6K have a repressing role on PLD2 gene expression as our lab has noted earlier (126) and an enhancement of mTOR/S6K due to PLD-derived PA has been indicated before (149).

As indicated in Figure 16, culturing cells in serum free media results in an increase in PLD activity. Figure 21B shows that serum-deprived cells in culture have an up-regulated EGFR/JAK3/PLD2-PA system and are especially sensitive to a combination of JAK3 and PLD2 enzymatic activity inhibitors (30 nM apigenin and 300 nM FIPI, respectively).
Figure 21. An upregulation of cell invasion by PLD2 and JAK3 overexpression is abrogated by Apigenin and FPII treatment. (A) Effectors of cell migration in the presence of 30 nM apigenin during cell invasion assay or from cells that had been silenced with dsRNA for 4 days prior to the invasion assay. In either case, cells were serum-deprived cells for 16 prior to cell invasion. (B) Effect of PLD activity inhibitor FPII (300 nM), the JAK3 inhibitor apigenin (30 nM), or a combination of the two, on cell invasion. The day before the experiment, cells were divided into two equal sets, one was kept cultured in normal media with serum and the
other was changed to a serum free media for 16 hours prior to cell invasion. Results are the means ± SEM from at least 3 independent experiments conducted in duplicate. The symbols * and # denote statistically significant (p<0.05) differences (increases or decreases, respectively) between samples and controls.
F. A EGFR→JAK3→PLD axis enhanced cancer cell invasion in cancer cells

I so far described a dependency of cell invasion by two kinases (EGFR and JAK3) and a phospholipase (PLD2). This multiple level provides regulatory flexibility and maximizes the aggressively invasive power of MDA-MB-231 cells. This is especially important in the absence of growth factors in serum or “survival mode”, coincidental with metastasis of these cells from the primary tumor to new locations. A model of the EGFR/JAK3/PLD2 system described in this thesis is presented in Figure 22.
Figure 22. The JAK3-PLD2 system underlies the highly invasive phenotype of cancer cells. A model depicting the results of this study. JAK3 switches from mild inhibition to robust promotion of cell migration through PLD2 during cell starvation, together with EGFR for maximal cancer cell survival.
VII. CONCLUSIONS AND DISCUSSION

(a) Relevant to specific aim 1

I observed the presence of the tyrosine kinases Fes and JAK3 in MCF10A (as a model of an untransformed cell line) and MDA-MB-231 (as a model of transformed cells), regulate one another and they also regulate PLD enzymatic activity. This inter-regulation is qualitatively and quantitatively different in the two cell lines investigated. The overall picture that emerged from my experiments is that in untransformed cells, both the protein expression levels and the enzymatic activity of the three signaling molecules (PLD2, JAK and Fes) are maintained at a low level. A complex inter-regulation equilibrium composed of activations and inhibitions keep PLD and the synthesis of PA in balance and a basal level. The situation is very different in transformed cells; both the protein expression levels and the activity of PLD2, JAK and Fes are up-regulated. The kinases activate PLD that is then synthesizing higher amounts of PA. Further, this PA feeds back positively on Fes that upon binding to the phospholipid, is further activated. Thus, I report the existence of a new signaling pathway involving PLD2, JAK3 and Fes that is heightened in transformed cells. As I also observed that cell proliferation is dependent on these three molecules and their inter-regulation, I concluded that this new pathway could be very important to promote abnormally high cell proliferation in cancer cells.

Several experiments were carried over to elucidate this new pathway. They were divided between (a) the effect of kinases on the lipase, as the effect of JAK3 on PLD2 and Fes on PLD2 and (b) the effect of the two kinases amongst themselves.

I observed the presence of JAK3 and Fes in MCF10A and MDA-MB-231 cells, and identified residues through which they are interacting with PLD2 and one another. Fes interacts
with PLD2 through the Y415 residue in MCF10A cells, wherein it phosphorylates that residue and induces activation. In MDA-MB-231 cells, the SH2 domain becomes more important, and interaction between Fes and PLD2 ceases when this domain is disrupted. In MCF10A cells, this domain is inconsequential, and interaction can still occur, whereas the converse is true in MDA-MB-231 cells with the Y415 mutant. In PLD2, the two tyrosine residues Y169 and Y179 in the N-terminal PX domain are situated within the YxN motif that is a canonical target recognized by proteins that possess SH2 domains. The SH2 domain binding increases PLD2 activity in cancer cells, and also contributes to heightened Fes activity, due to the fact that binding of the SH2 domain stabilizes kinase activity of Fes (35) and creates a positive feedback loop which re-stimulates PLD2. The binding of Fes to the SH2 binding domain of PLD2 may be due to hyper-phosphorylation of PLD2 thanks to EGF-R overexpression in cancer cells. In my results, I demonstrated that SH2i Fes binds more in MCF10A than wild-type, which may be due to heightened exposure of whatever site binds Y415. Loss of Y415 can be compensated by the SH2 binding in cancer cells. Future studies should examine the binding site of Fes to Y415 on PLD2, as it is not known at this time.

Additionally, I demonstrated that silencing JAK3 has an effect on PLD2 activity; PLD2 activity is increased in MCF10A cells upon silencing of JAK3 and decreased MDA-MB-231 cells, again highlighting the difference in regulation between the two proteins. PLD2 has been previously shown to be abundant in cancer cells that promote cell growth (59). Targeting PLD2 enzyme lipase activity with selective inhibitors has also been shown to have tremendous effects on decreasing metastasis and invasion (60). I have indicated here that PLD2 is controlled through both the JAK3 and Fes tyrosine kinases. JAK3 acts differentially in cancer cells, where it acts a positive regulator through the SH2 domain. Fes likewise shows a more dramatic effect in
positive regulation of PLD2 in cancer cells than normal cells (MCF10A). Taken together these differential interactions indicate why PLD2 is up-regulated, and helps to explain why the cancerous phenotype of MDA-MB-231 cells exists.

I additionally examined Fes with context to phosphatidic acid, using a PA sensor plasmid that fluoresced upon binding of PA. This allowed for the characterization of sub-cellular localization of PA. In MCF10A cells, PA is sequestered the nucleus, where it is present in tightly contained vesicle structures. Upon stimulation of cells with EGF, the PA is released and becomes cytosolic. In MDA-MB-231 cells, the PA is perinuclear, and is not present in the nucleus. PA is also more abundant in MDA-MB-231 cells, possibly due to PLD2 hyperactivity. Upon stimulation with EGF, PA in MDA-MB-231 cells becomes cytosolic, similar to what was observed in MCF10A cells. Next I utilized immunochemistry to examine sub-cellular localization of Fes with context to PA. I found that MCF10A has PA and Fes co-localize in similar fashion to PA, becoming cytosolic upon stimulation with EGF. There is a more pronounced co-localization in MDA-MB-231 cells, especially with stimulation of EGF. This suggests that the EGF pathway induced the two to co-localize within the cytosol where they may play some part in further interactions with JAK3 or PLD2. Overall it indicated that Fes co-localizes with and potentially binds Fes. I next examined Fes and JAK3 activities with context to phospholipids. I examined PA alongside phosphatidylinositol 2,4,5 bisphosphate and phosphatidylcholine as controls. PA showed the most marked increase in Fes activity, while no increase in JAK3 activity. PIP2 increased Fes activity, but not to the extent that PA did.

Phospholipids seemed to have little to no effect on JAK3 activity. I then examined Fes activity with varying amounts of PA in both MCF10A and MDA-MB-231 cells. Again, I found a
differential regulation of PA interaction, with PA increasing activity optimally at about 100 nM in MDA-MB-231 cells. No change was seen in MCF10A cells. It is possible that this is due a change in the exposure FX domain, which is known to interact with PA. It is known that Fer, a familial isoform of Fes, interacts downstream of PLD mediated PA generation through the FX domain which leads to heightened activity (53). It is possible that PA supplementation is fruitless in MCF10A cells due to its metabolism by a number of proteins, but in MDA-MB-231 cells PA remains steadily high due to heightened PLD2 activity and expression. Thus, the supplementation is enough to give Fes the boost required to up-regulate its activity. This finding suggests that the interplay between PLD2 and Fes is robust and is much more important in cancer cells.

My next objective was to identify interactions between JAK3 and Fes kinases themselves. I first silenced JAK3 in a number of cell lines and measured Fes kinase activity. In all transformed cell lines and MCF10A cells, increasing concentrations of siJAK3 decreased Fes activity, suggesting a positive regulatory role for JAK3 and Fes activity. I then overexpressed JAK3 in MCF10A and MDA-MB-231 cells, and found that Fes activity is heightened as a result in both cell lines. Additionally I also found that Fes inhibited JAK3. It is possible that the two interact through SH2 domains that both possess, however I speculate that they only phosphorylate one another without extensive binding.

Overall, I developed a model which has Fes, JAK3, and PLD2 with Fes down-regulating JAK3 and JAK3 down-regulating PLD2 in normal, untransformed cells. Fes interacts with PLD2 through the Y415 residue, which leads to stabilization of its activity. JAK3 down-regulates PLD2 through its kinase activity, and possibly through interaction through its SH2-L domain.
JAK3 may bind Y415 as well, which may be a site of inhibition. Fes may compete with JAK3 at this site, which provides stabilization by preventing inhibition by JAK3. Fes and JAK3 interact to down-regulate one another. In cancer cells the enhanced binding of Fes to PLD2 through its SH2 domain provides positive regulation as opposed to stabilization. This leads to an increase in PLD2 activity through Fes. Additionally, the PA generated by PLD2 in great abundance causes Fes to be stimulated, which feeds into a positive loop with PLD2. Furthermore, JAK3 acts positively on Fes in cancer cells, and Fes acts positively on JAK3. Due to the fact that the pathway is delicately regulated by competition between Fes and JAK3 in normal cells, the disruption of binding to PLD2 greatly disturbs the pathway generating a positive feedback loop, which leads to cytoskeletal reorganization and enhanced proliferation, contributing to the transformed phenotype.
CONCLUSIONS AND DISCUSSION (continued)

(b) Relevant to specific aim 2

Our laboratory has previously demonstrated that JAK3 is critical to cell chemotaxis in neutrophils (48). I initially observed that MDA-MB-231 cells did not rely on JAK3 for PLD2 mediated proliferation and cell invasion. However, upon serum starvation of the cells JAK3 became a positive signal in PLD2 activation and cell proliferation. Serum or nutrient (amino acids and glucose) removal also led to an increase in JAK3 activity that was reversed when those nutrients were added back to the serum. These experiments underscored the importance of JAK3 and PLD2 during times of nutrient deprivation, wherein survival signals are generated through the JAK3 PLD2 signaling axis.

An additional piece of evidence for the JAK3/PLD2 connection is given in this study by the use of the small molecule flavonoid inhibitor, apigenin. Apigenin does not directly inhibit PLD2, but rather inhibits JAK3 (that regulates PLD2). The decrease in PLD2 activity upon treatment with apigenin led us to believe JAK3 should be acting upstream of PLD2. I reasoned that apigenin’s inhibition of PLD2 is because JAK3 is unable to phosphorylate PLD2 in the presence of apigenin. There are other kinases indirectly participating in PLD2 activation, namely EGFR by means of JAK3 activation.

Apigenin is a powerful (IC$_{50}$=7-26 nM) inhibitor of invasiveness of cancer cells. In our hands, apigenin produces robust cell invasion inhibition at nanomolar concentrations, which is approximately 1000-fold less than the micromolar concentrations of apigenin (5-200 µM) used by others (1, 68). That apigenin would target JAK3 as a tyrosine kinase inhibitor is understandable in light of the work of Sudbeck et al., who has documented structural and
chemical features that facilitate binding of certain small inhibitor compounds to leukemic JAK3, which share a high level of similarity with apigenin (123).

I depict a model of apigenin’s disruption of the PLD2-JAK3 signaling axis in Fig. 22. The model highlights how JAK3 switches from inhibiting to promoting PLD2 activity during cell starvation for cancer cell survival and works together with EGFR for maximal cell migration, and this effect is interfered with by apigenin.

In breast cancer cells, PLD activity is increased during starvation, and Dr. Foster’s group has labeled PLD as a "survival signal" (148). An elevated PLD could prompt cells to migrate, presumably outside the inner part of a solid tumor (115), and it could serve a “nutrient sensor” in cooperation with mTOR (149). According to the results presented here, starvation that involves the effective removal of growth factors, lipids and other components from serum in it elevates PLD activity but precisely through JAK3. Thus, JAK3 is a key modulator of PLD-mediated cell invasion. In these highly invasive cancer cells, when starved, the elevated PLD activity will provide intracellular PA and still be able to activate mTOR/S6K leading to the activation of the translation machinery in conditions in which normal cells would typically be apoptotic or dead. The results presented here provide evidence of a novel EGFR/JAK3/PLD2 system that is activated under cell survival mode. This allows enhanced cell migration and invasion, via PLD2, which are reversed by apigenin. This study also highlights the usefulness of apigenin as a powerful inhibitor of cancer cell invasion.

In summary, this second part of the thesis elucidated a multi-layered activation of cell invasion by two kinases (EGFR and JAK3) and a phospholipase (PLD2) provides regulatory flexibility and maximizes the aggressively invasive power of MDA-MB-231 breast cancer cells.
This is especially important in the absence of growth factors in serum, coincidental with migration of these cells to new locations.
VIII. SIGNIFICANCE OF THIS STUDY

This project shows here a novel signaling pathway involving JAK3, FES and PLD2 in vivo, which participates in cell proliferation. This pathway was tested in a number of normal, untransformed cells (breast epithelial MC10A and COS-7 fibroblasts) and transformed, highly proliferative cells (MDA-MB-231 and MCF7 breast cancer cells, AML3D10 and HL60 myeloblasts). The experimental results here demonstrate the highly regulated control that JAK3, Fes and PLD2 exert on some aspects of intercellular signaling in cancer. Additionally, we present evidence that JAK3 and PLD2 communicate with one another through a pathway that is activated upon starvation of cells. This pathway accentuates invasiveness of cells during times of stress and also results in an increase of cell invasion. The pathway can be quelled by addition of the flavonoid apigenin, which inhibits JAK3 as well as EGFR (which activates JAK3). Both of these events down-regulate PLD2 activity and cell invasion. The results presented in this thesis demonstrate an overall pathway in which Fes, JAK3 and PLD2 participate to up-regulate proliferation of cells and enhance the invasiveness of breast cancer cells. These novel mechanisms of PLD2 signaling present potential therapeutic targets to prevent cancer progression.
IX. Reference


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