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Serum deprivation confers the MDA-MB-231 breast cancer line with an EGFR/JAK3/PLD2 system that maximizes cancer cell invasion

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Abstract

Our laboratory has reported earlier that in leukocytes, phospholipase D2 (PLD2) is under control of Janus Kinase-3 (JAK3), which mediates chemotaxis. Investigating JAK3 in cancer cells led to an important discovery as exponentially growing MDA-MB-231 human breast cancer cells, which are highly proliferative and metastatic, did not substantially use JAK3 to activate PLD2. However, in 2-h or 16-h starved cell cultures, JAK3 switches to a PLD2-enhancing role, consistent with the needs of those cells to enter a “survival state” that relies on an increase in PLD2 activity to withstand serum deprivation. Using a small-molecule tyrosine kinase inhibitor, the flavonoid apigenin (4',5,7-trihydroxyflavone), as well as RNA silencing, we found that the invasive phenotype of MDA-MB-231 cells is mediated by PLD2 under direct regulation of both JAK3 and the tyrosine kinase, Epidermal Growth Factor Receptor (EGFR). 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 (30 nM apigenin and 300 nM 5-Fluoro-2-Indolyl des-Chlorohalopemide [FIPI], respectively). Thus, 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.

INTRODUCTION

Neoplastic transformation and tumorigenesis have been associated with overexpression of PLD isozymes in cultured murine fibroblasts ¹, and high phospholipase D (PLD) activity has been documented in cancer cells ². Overexpression of either PLD1 or PLD2 results in the transformation of cells overexpressing a tyrosine kinase into a more malignant phenotype ³. There is also a requirement for an intact PLD1 catalytic activity in H-RasV12-induced transformation ⁴. PLD confers rapamycin resistance ⁵ and survival signals in human cancer cells with activated H-Ras or K-Ras ⁶. PLD has been implicated, among other oncogenes, in colorectal ⁷, renal ⁸ and gastric cancers ⁹, as well as melanoma ¹⁰. PLD is possibly involved in metastasis and can induce in vitro tumor cell invasion, while overexpression of PLD

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mediates matrix metalloproteinase (MMP) secretion¹³. It has been recognized that PLD2 has a powerful effect on signal transduction, adhesion, migration, invasion and metastasis in EL4 lymphoma cells¹⁴. The activation of this enzyme is found in lymphomas¹⁵. PLD also activates STAT3 that then activates the oncogenic kinase RET/PTC¹⁴ and is able to form protein-protein complexes with the EGF receptor¹⁶ or with Pyk2 and Src kinases¹⁷.

The MDA-MB-231 human breast cancer cell line is highly proliferative and metastatic and was obtained at the MD Anderson Cancer¹⁸. In vitro, the MDA-MB-231 cell line has an invasive phenotype, is able to grow on agarose, an indicator of transformation and tumorigenicity, and also displays a relatively high colony forming efficiency. In vivo, MDA-MB-231 cells are highly metastatic in nude mice. The MDA-MB-231 cell line functions as a valuable model for regulation of gene expression and cell proliferation in breast cancer and experimental metastasis. MDA-MB-231 cells, which have high levels of a mutant p53, has high levels of (PLD activity, which provides a survival signal in these cells when deprived of serum growth factors¹⁹.

As it is known that PLD can contribute to increased cell transformation and that MDA-MB-231 cells bear elevated PLD activity, we reasoned that these cells are ideal to study how the aggressive growth the highly invasive phenotype are regulated and if this regulation is dependent on PLD2. Also, characterizing small molecule inhibitors that could counteract this invasiveness phenotype of these breast cancer cells could be of great potential therapeutic benefit. Apigenin (4',5,7-trihydroxyflavone) is a plant polyphenol, flavonoid glycone derived from leafy vegetables that has an antibiotic function against Gram-negative bacteria. Apigenin has been found to inhibit cell proliferation by arresting the cell cycle at the G2/M phase²². Apigenin has also been shown to reduce cell viability, induce caspase-9- and caspase-3-dependent apoptotic cascades and elevate intracellular ROS levels in human HL-60 leukemic cells and HepG2 hepatoma cells²³. To date, there has been no analysis of the effects of apigenin on cell invasion of certain breast adenocarcinomas or non-small cell lung cancer and its mechanism of action.

We report here that the invasive phenotype of this cell line is mediated by PLD2 and is under the regulation of three specific tyrosine kinases. To dissect out the contribution of each kinase, we made use of the small-molecule inhibitor, apigenin. Apigenin inhibits PLD2-mediated cell invasion. Unexpectedly, Janus Kinase-3 (JAK3) was found to be inhibitory towards PLD2 activity in exponentially growing MDA-MB-231 cells. However, in 2-h or 16-h starved cell cultures, JAK3 switches to a PLD2-enhancing role, consistent with the needs of those cells to enter a "survival state" that relies on an increase in PLD2 activity.

We report here for the first time that a multi-layered activation of PLD2 by two kinases, JAK3 and EGFR, provides regulatory flexibility and maximizes the aggressively invasive power of MDA-MB-231 cells, especially in a serum-deprived or "survival" mode, as they invade adjacent tissue and migrate to new locations. Apigenin served to uncover the complex interregulation between the kinases and PLD2 and to indicate that this flavonoid is a powerful inhibitor of cancer cell invasion at the low nM concentration range via PLD2.

RESULTS

JAK3 is a major regulator of PLD2 and cell invasion

A laboratory survey on several cancer cell lines meant to assess the potency of JAK3 in PLD2-mediated cell invasion, extended our previous results of MTLn3²⁴ to human neutrophil-like differentiated HL-60 and AML-14-3D10 proleukemic cell lines, placing JAK3 as an activator of PLD2 in leukocytes for both endogenous (Fig. 1A, left and middle

group of bars) or overexpressed (Fig. 1B, left and middle group of bars) proteins. However, when we tested a new cell line, the highly invasive MDA-MB-231 human breast cancer cell line, we unexpectedly found that JAK3 did not activate PLD2 as it had for the other cancer cells. In fact, JAK3 provided a small, but statistically significant, inhibition of PLD2 activity for the endogenous protein (Fig. 1A, two bars on the right) or the overexpressed protein (Fig. 1B, two bars on the right).

We also silenced JAK3 with targeted dsRNA in HL-60 and AML-14-3D10 leukocytes and in MDA-MB-231 breast cancer cells. In the first two sets, JAK3 silencing led to a decrease in PLD2 activity whereas the opposite was true for MDA-MB-231 cells (Fig. 1C). Thus, figures 1A–C indicate a positive effect of JAK3 on PLD2 activity for differentiated leukocytes that could not be extrapolated to MDA-MB-231 cancer cells. A Matrigel cell invasion assay with these cells (exponentially growing) was performed next. Figure 1D indicates that cell invasion was slightly decreased with JAK3 overexpression and augmented with JAK3 silencing, paralleling the results of Figure 1B,C with PLD enzymatic activity, highlighting a correlation of these two events.

Serum deprivation induces JAK3 and PLD2 activity elevation

We reasoned that this potentially “anomalous” behavior of MDA-MB-231 cells with respect to the leukemic cells in terms of JAK3 activity could be a consequence of a differential way in which PLD is expressed in these highly invasive cancer cells, when normal growth versus starvation is considered. The Foster group have reported that serum withdrawal of cancer cells is a stress signal that leads to an augmentation of PLD2 activity²⁵. As presented in Fig. 2A, cell starvation by serum deprivation for 2 hr bears JAK3 as not being inhibitory of cell invasion anymore. At longer lengths of starvation, 16 hr, JAK3 definitely switches its effect on MDA-MB-231 cells to that of an activator of cell invasion which is now in line with the other cancer cells tested earlier (HL-60 and Eo-3D10, Fig. 1A,B,C). The reversal from a negative to a positive regulator makes sense as cancer cells in starvation would shut off negative signals (in this case JAK3) to get PLD2 to fully perform its “survival” role. This is in agreement with the idea advanced in⁶ and with JAK3 being defined for the first time here as having an important role.

We also observed a positive effect of serum deprivation on both PLD2 and JAK3 activities (Figure 2B,C). This further provides evidence for the close connection between the kinase and the lipase. Slight acidosis of the media (as it occurs when nutrients are exhausted) also has a similar effect albeit quantitatively less relevant, whereas culture of cells with OptiMEM, a “minimum” medium that lacks essential nutrients, negated the activation that could be somewhat rescued with adding back human serum at 15% (Figure 2B,C). PLD2 and JAK3 activities appear to go hand in hand as they responded similarly to the different experimental treatments (Figure 2B,C). Thus, serum-deprived MDA-MB-231 cells in “survival mode” would make full use of JAK3 to ensure maximal activation of PLD2 and the cell invasion response perhaps to facility the migration to a more growth-permissive environment as PLD2 has been shown to be necessary for cell migration¹⁵. Based on this, subsequent experiments were performed in 16-hr serum deprived cells.

The small molecule inhibitor apigenin allowed dissecting out the inter-regulation between JAK3 and PLD2

To more comprehensively study this new role of JAK3 in governing PLD2 activity as it relates to cell invasion of cancer cells, we made use of three different tyrosine kinase inhibitors, the flavonoids apigenin (4',5,7-trihydroxyflavone) and genistein and the quinazoline Janex-1. Fig. 3A shows that apigenin is an excellent inhibitor of JAK3 activity in vivo when compared to the two other tyrosine kinase inhibitors, genistein and Janex-1.

Apigenin induces >60% inhibition at a concentration of 30 nM and >80% at a concentration of 100 nM. Since apigenin inhibited JAK3 in cells, we next investigated if (a) this could translate into inhibition of cell invasion through an extracellular matrix, such as Matrigel in cell invasion assays, and if (b) the results on MDA-MB-231 could be extrapolatable to other cancer cells.

Results shown in Fig. 3B–D indicate that apigenin is a powerful inhibitor of cell invasion not only for MDA-MB-231 but also for two other cell lines tested (small lung cancer H1299 and murine breast cancer MTLn3). The IC₅₀'s are: 25 nM, 45 nM and 7 nM for MDA-MB-231, H1299 and MTLn3, respectively. Having established a robust inhibitory effect of apigenin in the three cancer cell lines, we went back to the subject of this study, MDA-MB-231, and asked if cell invasion was inhibited depending on the time of exposure. Fig. 4A,B indicates that apigenin (at 3 nM) inhibits both the rate (between 3–12 hours) and the extent of cell invasion in response to EGF through Matrigel (Figure 4B). Thus, apigenin, at nanomolar concentrations, is a powerful inhibitor of cancer cell invasion.

JAK3 is upstream PLD2 in signaling and positively modulates it

Since apigenin inhibits JAK3 activity as well as cell invasion, the next logical step was to investigate the regulatory relation between PLD2 and JAK3 as well as with other putative kinases, particularly EGFR as the growth factor used in this study to activate MDA-MB-231 cells is EGF. Figure 5A shows that apigenin robustly inhibits JAK3 but it also affects EGFR, although at a lesser extent than it does JAK3. Dose-response curves indicate that apigenin inhibits the two kinases with IC₅₀ of ~1 nM for JAK3 and ~20 nM for EGFR, with maximal inhibitions of ~80% and ~50%, respectively. Next, we measure PLD activity when cells are subjected to overexpression of tyrosine kinases EGFR and JAK3 constructs and the effect of both apigenin inhibition and JAK3 silencing (the latter as a more direct test of JAK3 than apigenin that can also inhibit EGFR, Figure 5A). Figures 5B,C and D indicate that: (a) JAK3 (as well as EGFR) overexpression augments endogenous PLD2 activity in MDA-MB-231 cells; (b) PLD activity is maximal when PLD2 is co-expressed along with JAK3 or with EGFR, which are negated by dsRNA silencing; and (c) apigenin does not inhibit PLD2 activity directly but it negates the increasing effect it receives from JAK3 (but slightly less from 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.

Activation of PLD in a JAK3-PA survival mode

As EGFR was another kinase that we had to consider after JAK3, we next asked if Ser/Thr kinases would be implicated. As models, we used mTOR and S6K for which our lab has expression plasmids. We observed that PLD2, and all 4 kinases JAK3, EGFR, mTOR and S6K are each capable of enhancing cell invasion at different degrees (Figure 6A) with the following hierarchical order: PLD2 > EGFR > JAK3 > S6K ≈ mTOR. Even though apigenin does not inhibit PLD2 directly, it inhibits cell migration as demonstrated above, because of its effect on JAK3. As expected, Ser/Thr kinases like mTOR or S6K were not affected by apigenin. As noted, both mTOR and S6K have a repressing role on PLD2 gene expression²⁶ and an enhancement of mTOR/S6K due to PLD-derived PA has been indicated before²⁷.

As indicated in Figure 2, culturing cells in serum free media led to an increase in PLD activity. Figure 6B shows that serum deprived cells in culture have an upregulated EGFR/JAK3/PLD2-PA system and are specially sensitive to a combination of JAK3 and PLD2 enzymatic activity inhibitors (30 nM apigenin and 300 nM 5-Fluoro-2-Indolyl des-Chlorohalopemide (FIPI), respectively). Thus, 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 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 study is presented in Figure 6C.

DISCUSSION

Our laboratory has shown previously the importance of JAK3 for cell chemotaxis in neutrophils²⁸. The initial observation for this study was that exponentially growing MDA-MB-231 human breast cancer cells, which are highly proliferative and metastatic, did not use JAK3 substantially to activate PLD2 during cell invasion (in fact, a small inhibition was observed). However, in 2-h or 16-h starved cell cultures, JAK3 switches to a PLD2-enhancing role, consistent with the needs of those cells to enter a “survival state” that relies on an increase in PLD2 activity to withstand serum deprivation. Serum or nutrient (amino acids and glucose) removal led to an increase in JAK3 activity that was reversed when those nutrients were added back to the reaction. These experiments also pointed at a profound connection between JAK3 and PLD2 in cancer cells (JAK3 being upstream of the lipase in cell signaling circuits).

A second piece of evidence for the JAK3/PLD2 connection is given in this study by the use of the small molecule flavonoid inhibitor, apigenin, that even though it does not directly inhibit PLD2 it does robustly inhibit PLD2-led cell invasion. We reasoned that this is because JAK3 is unable to phosphorylate PLD2 in the presence of apigenin. There are other kinases participating in PLD2 activation, chiefly EGFR by means of activating JAK3. Apigenin is a very strong ($IC_{50}=7-26$ nM) inhibitor of cancer cell invasion. 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 μ M) used by others. 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 apigenin³¹.

A model of the mechanism of JAK3-PLD2 interaction that is disrupted by apigenin is depicted in Fig. 6E. 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 the Foster group has labeled PLD as a “survival signal”²⁵. An elevated PLD could prompt cells to migrate, presumably outside the inner part of a solid tumor³² and it could serve a “nutrient sensor” in cooperation with mTOR²⁷. According to the results presented here, starvation that involves the effective removal of growth factors, lipids and other components from serum in itself 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.

MATERIALS AND METHODS

Reagents

DMEM was from Mediatech (Manassas, VA); α -MEM, Opti-MEM, Lipofectamine, Plus reagent and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA); matrigels were from BD Biosciences (San Jose, CA); hematoxylin was from Ricca Chemical Co. (Arlington, TX); apigenin (4',5,7-trihydroxyflavone) was from Sigma (St. Louis, MO); 5-Fluoro-2-Indolyl des-Chlorohalopemide (FIPI) was from Cayman Chemical (Ann Arbor, MI); purified recombinant human JAK3, genistein and Janex were from Millipore (Billerica, MA) and JAK3 tide synthetic peptide substrate was from (Fremont, CA); [^3H]-butanol was from American Radiolabeled Chemicals (St. Louis, MO); [^{32}P]- γ ATP was from Perkin-Elmer (Waltham, MA); ECL reagent was from GE Healthcare (Piscataway, NJ); EGF was from Peprotech (Rocky Hill, NJ) and siControl and siJAK3 were from Applied Biosystems (Foster City, CA).

Cells and cell culture

MDA-MB-231 and HL-60 cells were obtained from ATCC. MCF-7 and H1299 cells were a gift from Dr. Steven Berberich (Wright State University), MTLn3 cells were a gift from Dr. Jeffrey Segall (Albert Einstein College of Medicine) and Eo-310D cells were a gift from Dr. Michael Baumann (Wright State University). MCF-7, MDA-MB-231 and H1299 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), while MTLn3 cells were cultured in α -MEM supplemented with 5% (v/v) FBS. Promyelocytic leukemic HL-60 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 1.0 $\times 10^6$ /ml.

Cell invasion assays

Cells were serum-starved for 2 hr and resuspended at a concentration 1.5 $\times 10^6$ 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 μ l cells were applied to the upper chambers of 8 μ m PET matrigels (24-well format) with a 6.5 mm diameter membrane. Final concentration of chemoattractant used was 0 or 3 nM EGF in 500 μ l of chemotaxis buffer placed in the lower wells of 24-well plates. Cell (MDA-MB-231, MTLn3 and H1299) invasion assays were incubated for 6 hr, unless otherwise indicated, 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 20 $\times$ objective and a 10 $\times$ ocular.

Gene Overexpression and Silencing

The protocol for overexpression involved transfection of PLD2, JAK3, EGFR (or mTOR and S6K) plasmid DNA into MDA-MB-231 cells using Transit-LT1 (Mirus, Madison, WI) and DMEM cell culture media containing 10% fetal bovine serum (FBS) for 48 hr at 37 °C. The protocol for silencing involved transfection of 300 nM siJAK3 into MDA-MB-231 cells using Transit siQuest (Mirus, Madison, WI) and DMEM cell culture media containing 10% FBS. Silencing reactions were allowed to incubate at 37 °C overnight at which time cells were washed and re-fed with complete media in the absence of antibiotics and were allowed to be silenced for a total of 72–96 hr. A negative control for siRNA ("siControl") was also from Ambion. Neg-siRNA# 2 is a 19 bp scrambled sequence with 3' dT overhangs (sequence not disclosed by Ambion) certified not to have significant homology to any

known gene sequences from mouse, rat or human and causes no significant changes in gene expression of transfected cells after 48 hrs at the same concentration as the siRNA in test.

PLD Lipase assay

Purified baculoviral PLD2 was processed for PLD activity in PC8 liposomes and [³H]n-butanol beginning with the addition of the following reagents (final concentrations): 3.5 mM PC8 phospholipid, 45 mM HEPES (pH 7.8), and 1.0 μCi [³H]n-butanol in a liposome form, as indicated in ³³ to accomplish the transphosphatidylation reaction of PLD. Samples were incubated for 20 minutes at 30 °C with continuous shaking. 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 Assays

Samples containing 2×10^6 cells were incubated with the appropriate concentration of apigenin for 20 min in a 37°C water bath with vigorous shaking. After incubation with the inhibitor, cells were either mock-treated or treated with 3 nM EGF for an additional 14 min in a 37 °C water bath with vigorous shaking. After stimulation, cells were prepared as in ²⁸. After stimulation, cells were sedimented, 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 either 42 μM JAK3 tide substrate for the JAK3 kinase assay or EGFR synthetic peptide for the EGFR kinase assay to yield a 40 μl total kinase reaction volume. Reactions were incubated at 30 °C for 20 min and stopped by spotting 20 μl reactions onto 2 × 2.5 cm² pieces of P81 What man 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 *-fold* activation.

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.

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Revised Highlights of YJMBI63886

- Cell invasion of cancer cells relies on JAK3 and phospholipase D2 (PLD2).
- PLD2 and JAK3 activities are enhanced in serum-deprived cultures.
- The flavonoid apigenin is a strong inhibitor of PLD2, JAK3 and cell invasion.
- An EGFR/JAK3/PLD2 system exists under serum-deprivation or “cell survival mode”.
- The mechanism explains the aggressive invasiveness of MDA-MB-231 cancer cells.

Fig. 1. 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 μ g of recombinant JAK3 (Rec JAK3) 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 μ g DNA per condition) for two days. After this, they were lysed and incubated with 1 μ g

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 1×10^4 cells were placed on the upper or "insert" chambers of 6.5-mm, 8- μ m pore diameter, 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 $\pm$ 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.

Fig. 2. 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. To accomplish starvation 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 $5,940 \pm 50$ 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 $2,327 \pm 20$ cpm/mg protein. Results in this figure are the means $\pm$ 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.

Fig. 3. 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 μ l DMSO, final concentration) on MDA-MB-231 cells in vitro. Control cells received DMSO (1 μ l/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, for zero apigenin in both 0 nM EGF or 3 nM EGF, cells were incubated with DMSO alone (1 μ l/ml, final concentration). Results in each panel are the means $\pm$ SEM from 3 independent experiments conducted in duplicate.

B Hematoxylin-stained Matrigels

Fig. 4. 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 preincubated 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 $\times 20$.

Fig. 5. 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 from 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 $\pm$ 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.

Fig. 6. The JAK3-PLD2 system underlies the highly invasive phenotype of cancer cells
 (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 FIPI (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 in (A) and (B) are the means $\pm$ 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. (C) 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.