A NOVEL MOLECULAR RELATIONSHIP BETWEEN PARN AND PLD THAT, WHEN DEREGULATED, CONTRIBUTES TO THE AGGRESSIVE PHENOTYPE OF BREAST CANCER CELL LINES.

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

Master of Science

By

TAYLOR ELAINE MILLER

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Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Taylor Elaine Miller ENTITLED A novel molecular relationship between PARN and PLD that, when deregulated, contributes to the aggressive phenotype of breast cancer cell lines, BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Julian Gomez-Cambronero, Ph.D.
Thesis Director

Committee on Final Examination

Julian Gomez-Cambronero, Ph.D.

Madhavi Kadakia, Ph.D.
Chair, Department of Biochemistry and Molecular Biology

Michael Leffak, Ph.D.

Weiwen Long, Ph.D.

Robert E.W. Fyffe, Ph.D.
Vice President for Research and Dean of the Graduate School
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The removal of mRNA transcript poly(A) tails by 3′-5′ exonucleases is the rate-limiting step for controlled mRNA decay in eukaryotes. Poly(A)-specific ribonuclease (PARN) is one such exonuclease that degrades poly(A) tails, and although its in vitro activity is well-characterized, PARN’s patho-physiological roles in the cell are not well understood. Prior studies have found a possible role for PARN in cancer, in that PARN expression levels in human breast cancer tissues are often decreased compared to normal control tissues. Indeed, data mined from the ONCOMINE cancer array database showed that PARN is downregulated in patient invasive breast carcinoma samples compared to adjacent normal control tissue. Interestingly, phospholipase D (PLD), a cell-signaling molecule well known for promoting breast cancer cell growth, proliferation, and metastasis, was upregulated in these same breast carcinoma samples. PARN is known to target mRNA containing AU-rich elements (AREs) for degradation and although not identified in the literature as a target of PARN, PLD1 has large AREs in its 3’ UTR. Taken together, as the levels of PLD are elevated in breast cancer and PARN levels are decreased and PLD1 contains AREs that PARN may directly target, we hypothesized that a regulatory connection between PARN and PLD would exist. We investigated whether PARN regulates PLD and if PLD regulates PARN. In non-cancerous cell lines, such as in COS-7 and HMEC, we found that PARN downregulated the expression of both predominant mammalian isoforms of PLD, PLD1 and PLD2. This phenomenon was not observed in the breast cancer cell lines. We also investigated whether the converse was true, if PLD has any regulatory effect on PARN. We found that PLD and its catalytic product, phosphatidic acid (PA) in the exogenous form of dioleoyl-PA (DOPA), increased PARN expression and activity. PARN also co-localized with exogenously-added fluorescent PA in cells, as demonstrated by microscopy. However, DOPA did not bind to recombinant PARN protein in vitro (although PA species with saturated acyl chains did bind to PARN), suggesting that the effect of DOPA on regulating PARN
expression and activity is most likely through signaling downstream of PA. We hypothesized that the mechanism by which PARN downregulates PLD in non-cancerous cells would be absent or inhibited in breast cancer cell lines. We determined that PARN directly targets the AU-rich 3' UTR of PLD1 that initiates PLD1 mRNA degradation presumably through PARN deadenylation of the mRNA poly(A) tail. Furthermore, this effect was largely dependent on the presence of a miR-203 targeting site that immediately precedes a specific AU-rich element (ARE) in the 3' UTR of PLD1. Thus, a combination of PARN deadenylase activity and a putative presence of miR-203 synergizes in destabilizing PLD1 transcripts hampering translation. We found that this newly discovered mechanism of PLD regulation was absent in the highly aggressive MDA-MB-231 breast cancer cell line, which may explain the high expression of PLD in this and other breast cancer cell lines. We report herein the novel regulatory relationship between PARN and PLD that, when deregulated, contributes to the phenotype seen in the MDA-MB-231 breast cancer cell line.
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1. INTRODUCTION

mRNA Decay

The rates and balance of translation and mRNA decay are important factors for determining the quantity of protein expressed in the cell. Imbalance between these two processes can lead to either an overabundance of certain proteins (if translation occurs faster than mRNA decay) or the opposite case when mRNA decay occurs faster than translation. The shortening of eukaryotic poly (A) mRNA tails by 3’ to 5’ exonuclease is the rate limiting step of mRNA decay (Figure 1) and one of the most potent ways to repress mRNA translation and to induce transcript turnover (Funakoshi et al., 2007; Mitchell and Tollervey, 2000; Nousch et al., 2013; Wolf and Passmore, 2014). Adenylation and deadenylation control mRNA stability and thus gene expression necessary for not only basic cellular functions, such as development and cell differentiation, but also in the altered gene expression found in pathological conditions, such as chronic inflammation, cancer, and abnormal DNA damage response (Jalkanen et al., 2014; Mazan-Mamczarz et al., 2003; Zhang et al., 2015; Zhang et al., 2010). The two major eukaryotic deadenylases are the CCR4-NOT transcriptional regulatory complex and the PAN complex. A third deadenylase, poly(A)-specific ribonuclease (PARN), also degrades poly(A) tails from the 3’ end (Korner et al., 1998; Mian, 1997; Moser et al., 1997); however, its functional roles and regulation in vivo are not well-characterized.

PARN

PARN is a divalent metal (Mg2+) ion-dependent, poly(A)-specific, processive, 5’ GTP cap-interacting 3’ exonuclease (Martinez et al., 2000). Structurally, PARN consists of three distinct catalytic exonuclease sites within a nuclease domain, as well as two RNA-binding domains, termed the R3H and RNA recognition motifs (RRM) (Figure 2). While all domains have some degree of RNA binding capability, the R3H domain is most important for PARN dimer formation and stability, while the RRM domain influences 5’ GTP cap binding and PARN processivity (He and Yan, 2014). PARN functions in mRNA decay by cleaving poly(A) tails to yield adenosine monophosphate (AMP), which continues mRNA degradation in the 3’ to 5’ direction (Martinez et al., 2000). This
deadenylase not only prefers poly(A) compared to poly(U), poly(G), and poly(C), it also has been characterized as having a preference for mRNAs that have stretches of AU-rich elements (AREs) in their 3’ UTRs (Helfer et al., 2012; Korner and Wahle, 1997; Lai et al., 2003; Lin et al., 2007).

**Physiological Role of PARN**

Within the last two decades, rare autosomal recessive mutations in PARN resulting in PARN deficiency are involved in the development of severe forms of dyskeratosis congenita (DC), an inherited telomere disease characterized by shortened telomeres and a variety of disease phenotypes (Mason and Bessler, 2015; Stuart et al., 2015; Tummala et al., 2015). This is thought to be caused mainly due to the decrease in mature human telomerase RNA component (TERC), as PARN is essential in cleaving oligo(A) tails from immature TERC, thus preventing TERC’s degradation in the nucleus. Telomerase cannot function properly in the absence of mature TERC and progressive shortening of telomeres results (Moon et al., 2015; Shukla et al., 2016).

PARN targets a group of transcripts that code for cell migration and adhesion factors and also transcripts involved in p53, FAK and ERK/MAPK signaling, Fcγ receptor-mediated phagocytosis and BRCA1 DNA damage response (Devany et al., 2013; Lee et al., 2012). PARN is involved in the degradation of the oncogenic microRNA, miR-21-CA, allowing PTEN and p53 to function properly as tumor suppressors (Boele et al., 2014; Buiting et al., 1999; Copeland and Wormington, 2001). PARN can directly regulate the stability of transcripts for the oncogenes c-myc, c-fos, and c-jun. Its deadenylase activity ensures low levels of these mRNAs under normal conditions (Cevher et al., 2010; Devany et al., 2013; Maragozidis et al., 2012; Moraes et al., 2006). In addition, PARN overexpression in patient samples of squamous cell lung carcinoma (SCC) correlated with a significantly increased survival rate (Maragozidis et al., 2015). Taken together, these findings indicate a tumor suppressor-like function for PARN. As different types of cancers display variation in PARN expression, it is currently not well understood why PARN expression and/or activity becomes aberrant in cancer.
miRs

MicroRNA (miRNA, miR) are small (~22 nucleotide long) non-coding RNA molecules. After a series of maturation steps in the nucleus and cytoplasm, mature miRNA associate with proteins of the RISC (RNA Induced Silencing Complex). The miRNA loaded into the RISC will then bind to the miRNA compatible sequence in the 3' UTR (untranslated region) of a target mRNA. This association of the RISC and mRNA leads to inhibition of protein translation either by inhibiting ribosomal function or by inducing the degradation of mRNA (Figure 3).

miR-dependent PARN Targeting of mRNAs

PARN physically associates with Ago2 in the RISC (Zhang et al., 2015). Furthermore, PARN targeting of TP53 mRNA is dependent on the presence of TP53’s ARE in its 3’ UTR, as well as miR-504/miR-125b- targeting sites adjacent to the ARE. MicroRNA-125b-loaded RISC recruits PARN to TP53 mRNA causing subsequent TP53 transcript degradation (Devany et al., 2013; Zhang et al., 2015). The study by (Zhang et al., 2015) is so far the only published work showing that PARN acts via this mechanism, although this miR-mediated mechanism has also been demonstrated with other deadenylases, such as CAF1/CCR4/NOT1 and Pan2-Pan3 (Zhang et al., 2010).

PLD

Phospholipase D (PLD) is a membrane protein important for not only the structural integrity of cellular membranes but also for its role in cellular signaling through protein-protein interactions or through its product of enzymatic reaction, phosphatidic acid (PA) (Gomez-Cambronero, 2014). Although there are several isoforms of PLD, the most studied are PLD1 and PLD2. PLD hydrolyzes phosphatidylcholine (PC) into its lipid second messenger, PA, and also choline (Figure 4) (Wang et al., 1994).

Physiological Role of PLD

Pathways that are regulated by PLD have been extensively studied and cover vast pathways, such as apoptosis, autophagy, exocytosis, endocytosis, Golgi-ER trafficking, cell proliferation, and cell migration (Frohman, 2015). While important for maintaining
normal cell functions, both PLD1 and PLD2 are implicated in a variety of disease states with cancer being of main concern. Specifically, PLD1 has a role in controlling cell polarity, cell survival, and cell migration (Bruntz et al., 2014; Cheol Son et al., 2013; Ye et al., 2013; Zhong et al., 2003), while PLD2 overexpression is connected to breast cancer cell growth, proliferation, and metastasis and is a known cancer survival signal (Foster, 2004; Hatton et al., 2015; Henkels et al., 2013; Hui et al., 2006; Rodrik et al., 2005; Shi et al., 2007). The PA produced in PLD hydrolysis reactions is also highly mitogenic and is involved in chemotaxis and cell growth (Gomez-Cambronero, 2014). Currently, what controls the expression of PLD1 and PLD2 and why they are overexpressed in breast cancer are currently still unknown.

**PLD and miRs**

It has been shown that miRs 203a, 3619–5p, and 887–5p regulate PLD2 expression in MDA-MB-231 (highly aggressive breast cancer) and miR-203 can also regulate PLD2 in the U251 glioblastoma cell line (Chen et al., 2014; Fite et al., 2016; Fite and Gomez-Cambronero, 2016). Overexpression of these aforementioned miRs decreased not only the expression of PLD2 but also the invasiveness of the MDA-MB-231 cells. Conversely, exogenously added PA stimulated the expression of these same miRs (Fite et al., 2016; Fite and Gomez-Cambronero, 2016). Using TargetScanHuman bioinformatics analysis, miR-203 is predicted to target PLD1, which is currently unverified in the literature. It is worth considering the fact that the PLD1 3’ UTR has many possible AU-rich elements (AREs), one of which directly flanks the predicted miR-203 targeting site (Figure 5). It is possible that PARN may directly target PLD1 mRNA through this large ARE, and this mechanism may be miR-dependent (miR-203 specifically), as described earlier herein.

**Preliminary Data**

Before proceeding with any experiments, we wanted to first confirm that PLD gene expression is upregulated in human breast tumor samples by establishing the endogenous levels of PARN gene expression in these same samples. Using the Finak Breast dataset from the ONCOMINE cancer microarray database (Finak et al., 2008), we
determined that PARN was downregulated in invasive breast carcinoma stroma compared to adjacent non-cancerous breast stroma (Figure 6A), while gene expression of both PLD1 (Figure 6B) and PLD2 (Figure 6C) were significantly upregulated in the same dataset. These data suggest that PARN downregulation allowed upregulation of post-transcriptional events normally kept under its control, which could contribute to cancer development and progression in which PLD1 and PLD2 have already been implicated (Henkels et al., 2013; Kang et al., 2009; Kang et al., 2015; Park and Min do, 2011).

**Hypothesis and Specific Aims**

Based on the current available literature, we have identified several gaps in knowledge. First, the regulation of PLD1 and PLD2 is still at present poorly understood especially in the context of normal versus cancerous breast cells. It is unknown why PLD becomes or is maintained in an upregulated state in breast cancer. Second, regulation of PARN expression and also PARN's role in cancer are almost completely undefined, while no studies have focused solely on elucidating a clear role for PARN in cancers. Third, the direct mRNA targets of PARN as well as the mechanism by which PARN regulates these mRNA are poorly characterized. The miR-dependent mechanism by which PARN can regulate some of its ARE containing mRNAs remains controversial and understudied. Taken together with our preliminary data, we hypothesized that PARN regulates PLD1 and PLD2 expression under normal conditions and that this regulation could be attenuated in breast cancer cells. This hypothesis was tested in two distinct aims: 1.) To validate the regulatory relationship between PLD and PARN expression in a normal and breast cancer cell line; and 2.) To identify the mechanism by which PARN regulates PLD expression in normal versus breast cancer cell lines.
Figure 1. Deadenylation-dependent mRNA decay 3’-5’. An schematic illustrating the order of events in eukaryotic deadenylase-dependent mRNA decay with emphasis on PARN. Garneau et al. The highways and byways of mRNA decay. Nature Reviews Molecular Cell Biology 8; 113-126 (February 2007).
**Figure 2. Basic PARN structure.** PARN contains nuclease domains that carry out its exonuclease function (through 3 distinct exonuclease sites) as well as two RNA-binding domains, the R3H and RNA-recognition motifs (RRM).
Figure 3. MicroRNA mRNA post-transcriptional regulation mechanism schematic.

After exportation from the nucleus and further processing into mature microRNA in the cytoplasm, microRNA are loaded into the RNA-Induced Silencing Complex (RISC). The microRNA then facilitates the interaction between a complimentary sequenced target mRNA and the RISC to either 1.) cause the mRNA's degradation or 2.) repress translation through blockage of the ribosome resulting in less target mRNA translation into protein.
Figure 4. Reaction Scheme of PLD. PLD1 and PLD2 both hydrolyze Phosphatidylcholine (PC) into Phosphatidic Acid (PA) and free Choline. Rouzer, C.A. *Targeting Flu Through a Host Protein*. VICB Communications. August 20, 2014.
Figure 5. PLD1 3’ UTR ARE and miR-203 targeting site. The predicted miR-203 targeting site (ACAUUUCA) is colored red, while the adjacent ARE is colored orange.
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II. MATERIALS AND METHODS

Materials

Rabbit PLD2 (N-term) (Abgent Cat#: AP14669a, San Diego, CA), mouse PLD1 (F-12) (Santa Cruz Cat#: sc-28314), rabbit Pierce PARN (Thermo Scientific Cat#: PA5-30252, Pittsburgh, PA), rabbit beta-actin (Cell Signaling Cat#: 4970S, Danvers, MA), HRP-linked rabbit IgG (Cell Signaling Cat#: 7074S, Danvers, MA), and HRP-linked mouse IgG (Cell Signaling Cat#: 7076S, Danvers, MA) antibodies were used for western blots. For immunofluorescent microscopy, goat PARN (N-12) (Cat#: sc-47618) and donkey anti-goat IgG-R (Cat#: sc-2094) were purchased from Santa C’ruz Biotechnology (Dallas, TX). Antibodies were validated by the manufacturer. 1,2-dioleoyl-sn-3-phosphate (dioleolyl-PA; DOPA) (Cat#: 840875), 1,2-diarachidonyl-sn-glycero-3-phosphate (AraPA) (Cat#: 840886), 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) (Cat#: 830845), 1,2-dilauroyl-sn-glycero-3-phosphate (DLPA) (Cat#: 840635), 1-oleoyl-sn-glycero-2,3-cyclic-phosphate (Lyso-PA) (Cat#: 857328), 1,2-dioctanoyl-sn-glycero-3-phosphocholine (PC) (Cat#: 850315), L-a-Phosphatidylinositol-4,5-bisphosphate (PIP2) (Cat#: 840046), and 1-oleoyl-2-[(6-[[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphate (NBD-PA) (Cat#: 810175) were purchased from Avanti Polar Lipids (Alabaster, AL). Transit 2020 transfection reagent was from Mirus Bio (Madison, WI). ECL western blotting reagents were from GE Healthcare (Piscataway, NJ). QPCR reagents and enzymes were from Fisher Scientific/Life Technologies (Pittsburgh, PA). Plasmid DNAs used herein were as follows: pcDNA3.1-myc-PLD2-WT and pcDNA3.10-myc-PLD2-K758R were previously designed in our lab, pCMV6-myc-DDK-PARN-WT was from Origene (Cat#: RC207220, Rockville, MD) and this plasmid was the basis for site-directed mutagenesis to generate PARN-H377A. DharmaFECT 2 transfection reagent was purchased from Dharmaco/GE Healthcare (Cat#: T-2002-03). Ambion single siRNA were purchased from ThermoFisher Scientific (Pittsburgh, PA) and were as follows: 5 nM PARN siRNA (Cat#: AM16708 ID#: 11661), 5 nM PAN2 siRNA (Cat#:AM16708A ID#: 105177), and 50 µM Negative Control siRNA (Cat#: AM4611). Pooled siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX) and were as follows: 10 µM PARN siRNA (Cat#: sc-61297) and 10 µM control siRNA-A
(Cat#: sc-37007). Recombinant purified PARN (Cat#: TP307220) and PAN2 (Cat#:TP300573) were purchased from Origene. Dharmacon miRIDIAN Mimic Human hsa-miR-203a-3p (Cat#: C-300562-03) and miRIDIAN microRNA Mimic Negative Control #1 (Cat#: CN-001000-01-50) were purchased from GE Healthcare. The miR plasmids were both tagged to the GFP coding sequence. The empty vector negative control and miR-203 miExpress Precursor miRNA Expression Clone plasmids were from GeneCopoeia (Rockville, MD Cat #: HmiR0249-M04-B). The PLD1 and PLD2 3' UTR luciferase vectors were purchased from SwitchGear Genomics/Active Motif. Essential components purchased specifically for RNA IP were torula yeast RNA (Chem-Impex Int'l Inc. Cat#: 01625, Wood Dale, IL), polyuridylic acid potassium salt (MP Biomedicals Cat#: 102707, Solon, OH), ribonucleoside vanadyl complex (VRC) (New England BioLabs Cat#:S1402S), and QIAshredder columns (QIAGEN Cat#: 79654, Hilden, Germany).

Cell Culture

COS-7, MCF-7, and MDA-MB-231 cells were obtained from ATCC (Manassas, VA, USA). This repository has indicated that the cell lines were recently authenticated and tested negative for contamination. Human Mammary Epithelial cells (HMEC) and mammary epithelial cell growth media containing BPE, hEGF, hydrocortisone, GA-1000 and insulin were from Cell Applications, Inc. (San Diego, CA, USA). HMECs were cultured in TPP growth factor-treated flasks (Cat#:90075) and 6-well plates (Cat#:92406). COS-7, MCF-7, MDA-MB-231 cells were cultured in HyClone Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). All cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO₂.

ONCOMINE

Using the ONCOMINE cancer microarray database (www.oncomine.org) as an integrated data-mining tool, we compared the gene expression profiles of PARN and PLD2 in 49 human breast cancer stroma samples contrasted against 6 healthy breast stroma samples. We mined the Finak Breast dataset (Finak et al., 2008) that analyzed
subtypes of tumor stroma corresponding to good- and poor-outcome breast cancers from invasive breast cancer patients

**Dioleoyl-PA/ NBD-PA treatment**

“Super-Stock” 1 mM dioleoyl-PA was prepared with 1 mg of 1,2-dioleoyl-sn-3-phosphate, or NBD-PA, in 1.4 mL of “Super-Stock Buffer” consisting of 50 mg of Fatty Acid-free BSA per 10 mL of 1x PBS, pH 7.2. Dioleoyl-PA is a cell permeable form of phosphatidic acid (PA) (Lehman et al., 2007). This “Super-Stock” dioleoyl-PA was then sonicated on ice 2x 4 s each with a 4 s pause in between sonications. “Intermediate 100 µM liposomes” were then made using 25 µl of the “Super-Stock” dioleoyl-PA and 225 µl of Cell Starvation Media (DMEM + 0.1% bovine serum albumin), which were then used to prepare the final concentration of 30 nM (unless otherwise indicated by the figure legends) dioleoyl-PA used to incubate cells for the indicated times in various figures.

**Cell Transfection: Overexpression**

Cells were plated into 6-well plates in complete media and allowed to grow 12-24 h before transfection. The PLD2 plasmid used codes for the entire gene including the 3’ UTR, while the PLD1 plasmid excludes the 3’ UTR. All PLD and PARN overexpression transfections were accomplished using 2 µg of PLD1 or PLD2 plasmid and/or 1 µg of PARN plasmid, 300 µl Opti-Mem Serum-Free media, and 2 µl of Transit 2020 transfection reagent per 1 µg DNA. miR plasmid overexpression reactions consisted of 2 µg miR-203 mimic, 300 µl Opti-Mem Serum-Free media, and 2 µl of Transit 2020 transfection reagent per 1 µg DNA. miR mimic transfection reactions for luciferase assays consisted of 50 nM concentrations of miR-203 mimic, adequate Opti-MEM serum-free media, and the appropriate volume of DharmaFECT 2 transfection reagent based on the cell line (COS-7) and number of cells seeded per well (100,000) as recommended by the manufacturer. All reactions were incubated at room temperature for 20 min before being added drop-wise to the corresponding cells growing in complete media. Cells were then incubated at 37 °C in an incubator with a humidified atmosphere of 5% CO₂ for 24 h. Media was aspirated and fresh complete media added and cells
Cell Transfection: Silencing

Cells were plated into 6-well plates in complete media and allowed to grow 12-24 h before transfection. Both the single and pooled (3 siRNA each) PARN, PAN2, and negative control siRNAs purchased were diluted with the manufacturer’s included nuclease free water to a stock concentration of 50 µM. These siRNA solutions were then further diluted to 150 nM (yields 50 nM concentration for each siRNA contained within the pooled siRNAs) for use in all silencing experiments. Equal concentration of negative control siRNA was used in the negative control siRNA transfections. The siRNA reactions consisted of siRNA, 300 µl Opti-Mem Serum-Free media, and 5 µl DharmaFECT 2 transfection reagent. All reactions were incubated at room temperature for 20 min before being added drop-wise to the corresponding cells growing in complete media. Cells were then incubated at 37 °C in an incubator with a humidified atmosphere of 5% CO₂ for 24 h. Media was aspirated and fresh complete media added and cells allowed another 24 h of growth in the incubator before adding a booster of siRNA. Cells were harvested the next day.

Quantitative Real time PCR (qRT-PCR)

Total RNA was isolated from cells with the RNeasy minikit. RNA concentrations were quantified using the NanoDrop ND-1000 UV/Vis spectrophotometer and samples were normalized to 2 µg RNA. Reverse transcription was performed with 2 µg RNA, 210 ng random hexamers, 500 µM dNTPs, 84 units RNaseOUT, and 210 units of SuperscriptII reverse transcriptase and incubated at 42°C for 55 minutes. qRT-PCR reactions were run with 100 ng total input RNA, 1 µl (which contained 250 nM of the probe and 900 nM of the primers) of either FAM-labeled PARN (Hs00377733_m1 Cat#: 4331182) and/or FAM-labeled PLD2 (Hs01093219_m1 Cat#: 4351372) and/or PLD1 (Hs00160118 Cat#: 4331182) gene expression assay multiplexed with the FAM-labeled housekeeping genes Actin (Hs01060665_g1 Cat#: 4331182), GAPDH (Hs02758991_g1 Cat#: 4331182), and TATA-Binding protein (Hs00427621_m1 Cat#: 4331182). qRT-PCR conditions for the Stratagene Mx3000P were: 95°C for 3 min and then 40 cycles of
the next 3 steps: 30 s at 95°C, 1 min at 60°C, and then 1 min at 72°C. The “cycle threshold” Ct values were arbitrarily chosen from the linear part of the PCR amplification curve where an increase in fluorescence can be detected >10 S.E.M. above the background signal. ΔCt was calculated as: ΔCt = Avg. PLD Ct – Avg. Housekeeping Ct; and gene fold expression, as $2^{-(\Delta\Delta Ct)} = 2^{-(\text{experimental Condition } \Delta Ct - \text{Control } \Delta Ct)}$.

**miR expression measurement by Quantitative Real time PCR (qRT-PCR).**

The TaqMan microRNA Cells-to-CT kit (catalog no. 4391848; Life Technologies) was used to prepare these cell lysates according to the manufacturer’s protocol. RNA concentrations were determined using a NanoDrop, and samples were normalized to 60 ng/μl RNA. Reverse transcription was performed in a 15-μl reaction volume with 1 μg of RNA, 1.5 μl 10X RT buffer, 1 mM dNTPs, 3.8 units of RNase inhibitor, and 1 μl of Multiscribe reverse transcriptase, and the mixture was incubated in one cycle at 16°C for 30 min, 42°C for 30 min, and then 85°C for 5 min. Quantitative PCR's were run in a 20-μl reaction volume using 10 μl TaqMan master mix, 100 ng of total input RNA, and 1 μl of the relevant microRNA gene expression assay (FAM labeled) multiplexed with the housekeeping gene (U6). TaqMan miRNA primers and fluorescent probes were from Life Technologies. Quantitative PCR conditions for the Stratagene Cycler were 95°C for 10 min and then 50 cycles of the next 3 steps, i.e., 15 s at 95°C, 1 min at 55°C, and then 30 s at 72°C. The CT values were chosen from the linear part of the PCR amplification curve, where an increase in fluorescence can be detected at >10 standard errors (SE) above the background signal. ΔCT was calculated as follows: ΔCT = avg PLD CT - avg housekeeping CT. The gene expression fold change was calculated as $2^{-(\Delta\Delta Ct)} = 2^{-(\text{experimental condition } \Delta Ct - \text{control } \Delta Ct)}$.

**SDS-PAGE/Western Blotting**

COS-7, HMEC, MCF-7, and MDA-MB-231 cells were transfected as described previously in this materials and methods section with expression plasmids that are defined in the figure legends. Cell media was aspirated from the plates and cells washed 2x gently with 1x PBS. Cells were then lifted from the plates by incubation with the addition of 1 ml trypsin for no more than 5 min, collected into 1.5 ml conical snap-cap
tubes, and sedimented at 14000 rpm 4°C for 1 min. The supernatant was aspirated to waste and cell pellets were resuspended in Special Lysis Buffer (5mM HEPES, 1 µM leupeptin, 768 nM aprotinin, 100 µM sodium orthovanadate and 0.4% Triton X-100) (SLB). After sonication of the lysates, samples were resolved using SDS–PAGE and transferred to a PVDF membrane, followed by immunoblot analysis. Primary antibodies used were as follows: anti-PARN (1:2000 dilution), anti-PLD1 (1:500 dilution), anti-PLD2 (1:500 dilution), and anti-actin (dilution 1:3000), which was used as the equal protein loading control. Secondary antibodies used were anti-rabbit or anti-mouse conjugated to horseradish peroxidase (HRP) (1:3000 dilution). Results were visualized using ECL reagents.

**Coomassie Staining**

Approximately, 100 ng of purified recombinant PARN and PAN2 protein were electrophoresed on gels using a conventional SDS-PAGE protocol. The gel was then rinsed 3 times for 5 min each in purified DI water then incubated overnight in 20 mL of GelCode™ Blue Safe Protein Stain (Thermo Scientific, Cat #: 1860983) with gentle shaking. The gel was then destained using multiple distilled water rinses until the water remained colorless and the background of the stained gel was reduced to minimal staining.

**Immunofluorescence microscopy**

COS-7 cells were seeded onto sterilized glass coverslips placed in the bottom of 6-well plates and allowed 24 h to adhere. Cells were incubated for 30 min in 30 nM fluorescent NBD-PA. Media was aspirated from the cells and then gently rinsed 1x with 1x PBS. Cells were then fixed onto their coverslips using 4% paraformaldehyde for 10 min at room temperature. The cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temp and then incubated in IF blocking buffer (10% fetal calf serum (FCS), 0.1% Triton X-100, and 1X PBS) for 1 h at room temp. Endogenous expression of PARN was detected in samples using a 1:200 dilution of PARN anti-goat antibody in IF blocking buffer for 1 h at room temperature, followed by washing 3x in 1X PBS, and further room temperature incubation with a 1:200 dilution of TRITC-
conjugated donkey anti-goat secondary antibody blocking buffer for 1 h. After washing 3x in 1X PBS, nuclei were stained using a 1:2000 dilution of 4,6-diamidino-2-phenylindole (DAPI) in 1X PBS for 5 min at room temp. Coverslips were washed again 3x in 1X PBS and 1x in distilled autoclaved water. Coverslips were then mounted onto clean glass microscope slides using VectaShield mounting medium (Vector Laboratories Cat#: H-1000, Burlingame, CA), and cells were visualized using a Nikon 50 Eclipse epifluorescence microscope.

Lipid-Protein Overlay Assay

The method for preparing and detecting protein-lipid binding has previously been described (Dowler et al., 2002). In brief, lipids from Avanti Polar Lipids (Alabaster, AL, USA) were spotted onto a PVDF membrane. Lipids were dissolved in a 2.0:1.0:0.8 ratio solution of MeOH:CHCl3:H2O. Appropriate amounts of lipid were spotted onto the membrane. The membrane was blocked overnight with a 3% fatty acid–free BSA solution. The membrane was then incubated overnight with recombinant PARN protein. After protein incubation, the membrane was incubated overnight with PARN primary antibody and was then incubated with rabbit secondary antibody, which was detected using ECL reagents.

Luciferase Assay

Luciferase reporter assays were performed using the ActiveMotif LightSwitch Luciferase Assay kit (Cat #: 32031). The reporter vectors contained PLD1 or PLD2 3′ UTR region and a downstream RenSP luciferase region. The PLD1 3′ UTR luciferase plasmid was the basis for site-directed mutagenesis (performed by Mutagenex, Suwanee, GA) to generate five ARE mutants. Cells were transfected or co-transfected as previously described with either a miR mimic or plasmid DNA and a LightSwitch PLD1 3′ UTR vector, PLD2 3′ UTR vector, or PLD1 3′ UTR mutant construct in a 96-well plate for 36 h. LightSwitch Luciferase Assay Reagent was added to the wells, and the signal was measured on a luminometer. The luciferase signal for each condition was calculated and normalized to the negative control.
Mutant Luciferase Constructs

To investigate the importance of the miR-203 targeting site and ARE of interest in the 3’ UTR of PLD1 for PARN targeting, five new mutant PLD1 3’ UTR plasmids were created (Figure 7). The LightSwitch PLD1 3’ UTR plasmid was used as the basis for site-directed mutagenesis to generate these new mutants. One substitution mutant, PLD1 3’ UTR mutMIR, had the miR-203 ACAUUUCA targeting site at bp 3021 to 3028 of the full plasmid mutated to UGUAAAGU to disrupt miR-203 binding. Four deletion mutants were also created. PLD1 3’ UTR ΔMIR had the entire miR-203 ACAUUUCA targeting site deleted (bp 3021 to 3029 of the full plasmid). PLD1 3’ UTR Δ1/3ARE had approximately 1/3 of the ARE of interest deleted (bp 3550 to 3813 of the full plasmid). PLD1 3’ UTR Δ2/3ARE had approximately 2/3 of the ARE of interest deleted (bp 3290 to 3813 of the full plasmid). PLD1 3’ UTR ΔARE had the entire ARE of interest deleted (bp 3030 to 3813 of the full plasmid).

RNA IP

The RNA IP protocol was based on (Conrad, 2008) (Figure 8). COS-7, HMEC, MCF-7, and MDA-MB-231 cells were plated in 6-well plates and grown to confluency. Plates were then placed on ice and media replaced with ice cold 1X PBS and cells fixed via 250 mJ/cm² UV irradiation using the Stratalinker UV Crosslinker (Model 1800 Cat#:400071). Cells were then scraped with a rubber policeman, collected, pelleted, and resuspended in 1 mL ice cold 1X PBS. Samples were then centrifuged at 2400 x g for 1 min at 4°C and the supernatant decanted to waste. Approximately, 140 µL of SDS lysis buffer was added and pellets gently mixed. Samples were then heated in a 65°C water bath for 5 min and subsequently incubated on ice for 3 min. Approximately, 560 µL of ice cold RIPA correction buffer was added to each sample and samples passed through individual QIAshredder spin columns twice by centrifugation at 16,000 x g for 1 min each. The resulting pellets were sedimented 3 more times at 16,000 x g for 10 min each, transferring the supernatant to new tubes each time and discarding the pellets. Approximately, 200 µL of PARN antibody-bound Protein G agarose beads were added to each sample and rotated at 4°C for 2 h. Beads were allowed to settle out of solution at room temperature for 5 min and supernatants gently aspirated to waste. Beads were
resuspended in 400 µL ice-cold RIPA buffer, centrifuged at 850 x g at room temp for 1 min. Beads were allowed to settle out of solution at room temp for 5 min. This RIPA buffer step was repeated 3 more times for a total of 4 RIPA buffer washes. Beads were resuspended in 200 µL RIPA buffer, 300 µl Proteinase K solution added, and then placed in a 37°C water bath for 1.5 h. To this mix, 30 µl of 3 M sodium acetate (pH 5.2), and 350 µL Phenol:chloroform:isoamyl alcohol, 25:24:1 (pH 7.5; PCA) were added, vortexed for 10 s, and then centrifuged at 16,000 g at room temp for 5 min. The aqueous phase of each sample was transferred to tubes containing 900 µl of 100% ethanol, incubated on dry ice for 15 min, and centrifuged at 16,000 x g for 15 min at room temp. Pellets were washed with 70% ethanol and centrifuged at 16,000 x g for 5 min at room temp. Ethanol was aspirated from the pellets that were then allowed to air dry. Pellets were resuspended in 20 µl RNase-free water and 20 µl of 2X DNase solution added. Samples were incubated at 37°C for 45 min then 180 µl of G-50 buffer, and 200 µl PCA added, vortexed 10s, and centrifuged at 16,000 x g for 5 min at room temp. The aqueous layer was transferred to tubes containing 500 µl of 100% ethanol and centrifuged at 16,000 x g for 15 min at room temp. The ethanol was aspirated and the pellet resuspended in 20µl of RNase-free water, and the RNA concentration was measured using the NanoDrop. Samples were then used to backtranscribe cDNA and perform qRT-PCR as described in these methods.

**PLD Activity Assay**

Cell lysates were 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, to accomplish the transphosphatidylation reaction of PLD, and were incubated for 20 min at 30 °C with continuous shaking. Reactions were stopped with the addition of 0.3 ml ice-cold chloroform/methanol (1:2), and lipids were isolated and resolved by thin layer chromatography. The amount of [3H]-phospho-butanol ([3H]-PBut) that co-migrated with PBut standards (Rf=0.45–0.50) was measured by scintillation spectrometry.
PARN Activity Assay

The PARN activity assay was based on (Shyu et al., 1991; Wilson and Treisman, 1988) with some modifications. Cell lysates were treated with 10μl/mL Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Cat #: 78440) and used in deadenylation reactions of [32P]-γATP radiolabeled A15 substrate. The A15 substrate and poly(A) ladder (A2, A5, A10, A15, and A30 mixed together) were 5' radiolabeled by T4 Polynucleotide Kinase (T4PNK). The positive control was recombinant purified PARN protein and the negative control was A15 substrate alone. Reaction products were subjected to autoradiography. Deadenylation is evidenced by a greater mobility of radiolabeled spots, the appearance of smears versus the negative control of A15 alone, and a lighter signal. This was quantified with the following formula:

\[ \text{PARN Activity} = \text{Migration (in mm)} \times \frac{\text{A10}}{\text{A15}} \times \text{Area of the band} \times \frac{1}{\text{density of band}} \]

Statistical Analysis

Results are expressed as means ± Standard Error of the Mean (SEM). The difference between means was assessed by t-test calculated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA) and P values of <0.05 were taken as significant. In the figures, the (*) symbols above bars denote statistically significant increases between samples. The (#) symbols above bars denote statistically significant decreases between samples.
Figure 7. Schematic of PLD1 3’ UTR LightSwitch Luciferase plasmid and its mutants. The luminescent reporter gene (RenSP) is a small *Renilla reniformis* luciferase protein that lacks dependence on ATP, unlike firefly luciferase, and produces light at 480 nm. This region is colored green in the schematic above. The miR-203 targeting site within the 3’ UTR is indicated by a thick blue box (light blue for the mutated site) at 2659 to 2666 bp in each schematic, except for the ΔMIR construct in which the miR-203 site is deleted. The ARE portion of the 3’ UTR is colored orange and is progressively shorter in each of the three ARE mutants. Δ1/3ARE has the last third of the ARE removed. Δ2/3ARE has the last two-thirds of the ARE removed. ΔARE has the entire ARE removed up to the miR-203 targeting site.
Figure 8. Pictorial representation of the RNA I.P. protocol. Plated cells are cross linked using UV irradiation and then collected. After several treatments with various buffers and centrifugation, whole cell lysate from these cells is added to Protein G agarose beads coated with IgG antibody specific for the protein of interest. When the whole cell lysate is added to these antibody-bound beads, the protein of interest will be bound to the antibody and pulled out of solution. By the end of the RNA I.P. process, DNA and protein are degraded, leaving behind only the RNA that was pulled down in complex with the protein of interest.
III. RESULTS

Differential expression of PARN, PLD1, and PLD2 in breast cancer cell lines compared to 'normal' cell lines.

A molecular connection between deadenylases and PLDs has not been investigated to date. Therefore, we determined the endogenous gene expression of PARN, PLD1, and PLD2 in COS-7 fibroblasts (one example of a normal cell line) and HMEC (a non-cancerous human mammary epithelium cell line), as well as in the breast cancer cell lines MCF-7 (low aggressive) and MDA-MB-231 (highly aggressive). MCF-7 and MDA-MB-231 cells had much higher PLD1 and PLD2 but lower PARN gene expression compared to the two normal cell lines (Figure 9). This results was also generally reflected in the protein expression results as well, although with some degree of variation (Figure 10). The fact that MCF-7 and MDA-MB-231 cells had more PLD expression than non-cancerous cells was in accordance with previous studies (Fite et al., 2016; Fite and Gomez-Cambronero, 2016). The observation that MCF-7 and MDA-MB-231 cells also had less PARN expression than the non-cancerous cells was in agreement with the Finak Breast data-mining results (Preliminary Data, Figure 6), and as such, we moved forward with the investigation of the possible relationship between PLD and PARN in these cell lines of interest.

PARN negatively regulates PLD2 expression in normal cells and PLD2 positively regulates PARN.

First, we sought to elucidate the regulatory relationship between PARN and the PLD2 isoform. When PARN was silenced in the normal cell line (Figure 11A, quantification B-C), PLD2 protein increased above that of the mock and negative siRNA-transfected cells, while PARN protein decreased. Similar results were observed in PARN-silenced cancerous MCF-7 cells (Figure 11D, quantification E-F). These results indicate that PARN has a strong negative regulatory effect on cellular PLD2 levels.
Figure 9. PLD and PARN are differentially expressed in breast cancer versus non-cancerous cell lines. Quantitative RT-PCR results for endogenous gene expression of (A) PARN, (B) PLD1, and (C) PLD2 in the cell lines of interest: COS-7 (green monkey kidney fibroblasts), HMEC (non-cancerous human mammary epithelium), MCF-7 (low aggressive human breast cancer), and MDA-MB-231 (high aggressive human breast cancer). Results are relative to three housekeeping genes: TBP, GAPDH, and Actin. Error bars are means ± s.e.m. The differences between the means were assessed by t-test. *P<0.05 **P<0.01 ***P<0.005 and ****P<0.001, significant increase between samples; #P<0.05, significant decrease between samples.
Figure 10. PLD and PARN protein expression in the cell lines. Western blot results for endogenous protein expression of PLD1, PLD2, and PARN in the cell lines of interest. The PLD1 blots come from two different gels, as many cell lines were run at one time, therefore this panel is split to show the results of the COS-7 and HMECs, which were run on the same gel together, as well as the MCF-7 and MDAs, which were run on another separate gel together. Actin is used as equal protein loading control. Images are representative of n=4.
Figure 11. Silencing of PARN increased PLD2 protein expression. Cells were treated with transfection reagents only (Mock) or silenced with siRNA-negative control (Neg.) or with siRNA for PARN as indicated. Four days post-transfection, lysates were used for protein expression analyses. (A-C) Protein expression for COS-7 cells and (D-F) for MCF-7 cells. Western blots are presented in A and D and the densitometry of PARN and PLD2 bands are shown in COS-7 cells (B-C) and MCF-7 cells (E-F) relative to their corresponding Actin. Actin was used as the equal protein loading control. Western blot images are representative of n=3. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05, significant increase between samples and controls; #P<0.05, significant decrease between samples and controls.
Differential effects of PARN+PLD2 co-expression in normal cells versus breast cancer cells.

Since a robust signaling interaction between PARN and PLD2 seems to exist in cells, we next transfected both types of cells with a combination of expression plasmids coding for PARN or PLD2 proteins. Co-expression of PARN+PLD2 in COS-7 cells led to an overabundance of PARN protein expression with a concomitant abrogation in PLD2 protein expression (Figure 12A). This would indicate that in COS-7 cells, PARN is able to control PLD2 levels to mock level even when PLD2 is overexpressed. Unexpectedly, in the MCF-7 breast cancer cells, both PARN and PLD2 protein co-overexpression augmented the expression of each other (Figure 12B), which is completely the opposite result seen in COS-7 cells. The results of qRT-PCR assays to measure gene expression in these same conditions are shown for PLD2 in Figure 13A-B and for PARN in Figure 13C-D, and reinforce the protein expression results.

To further validate the co-overexpression results in Figure 12 and 13, we performed a PLD activity assay in samples treated mock or overexpressed PLD2, PLD2 + PARN wild-type (WT), or PLD2 + catalytically inactive PARN-H377A. As shown in Figure 14, similar to the protein and qRT-PCR results, overexpression of PARN-WT with PLD2 was able to decrease PLD activity compared to the PLD2 overexpressed sample alone. Of significance is the observation that the catalytically inactive PARN was not able to reduce PLD2 activity. Since PLD2 levels are naturally high in cancer cells (Hui et al., 2006; Shi et al., 2007; Zheng et al., 2006), our results indicate that not only is PARN expression low in breast cancer cells, its ability to seemingly regulate expression of proteins, such as PLD2, is dysregulated. This phenomenon would be advantageous for a cancer cell, as PLD2 is implicated in cell invasion and cancer metastasis (Chen et al., 2012; Henkels et al., 2013; Knoepp et al., 2008).
Figure 12. PARN overexpression keeps PLD2 protein near Mock expression levels in COS-7, but not the MCF-7 cell line. (A) Western Blot results for PARN and PLD2 protein expression in COS-7 and (B) MCF-7 cells showing mock transfection alone, PLD2 overexpressed, PARN overexpressed, or PLD2 + PARN co-overexpressed. Actin was used as equal protein loading control. Images are representative of n=3.
Figure 13. PARN co-overexpression with PLD2 partially negates PLD2 gene overexpression, an effect not seen in the MCF-7 cell line. (A) PLD2 gene expression in COS-7 and (B) MCF-7 cells with mock transfection alone, PLD2 overexpressed, PARN overexpressed, or PLD2 + PARN co-overexpressed as measured via qRT-PCR. (C) PARN gene expression in COS-7 and (D) MCF-7 cells with mock transfection alone, PLD2 overexpressed, PARN overexpressed, and PLD2 + PARN co-overexpressed as measured via qRT-PCR. Results are relative to Actin housekeeping gene. Error bars are means ± s.e.m. The difference between means was assessed by t-test. *P<0.05 **P<0.01 and ***P<0.005, significant increase between samples; #P<0.05, significant decrease between samples.
Figure 14. Wild-type PARN co-overexpression with PLD2 partially returns PLD activity to near mock level. PLD activity in COS-7 cells treated with transfection reagents alone (Mock), PLD2 overexpressed, PLD2 + PARN-WT overexpressed, or PLD2 + PARN-H377A overexpressed. Error bars are means ± s.e.m. The difference between means was assessed by t-test. *P<0.05, significant increase between samples and control; #P<0.05 significant decrease between samples.
**PLD2 catalytic activity is required to exert an effect on PARN gene expression.**

We next investigated if the catalytic activity of PARN and PLD2 are required to influence the expression of each other. At least in COS-7, PARN overexpression decreased PLD2 gene expression. Using the PARN deadenylase-inactive mutant, PARN-H377A, we observed that the ectopic expression of this inactive mutant PARN also decreased expression of PLD2 to a similar degree to that of wild-type PARN, even though as shown previously in Figure 14, the PARN-H377A mutant does not significantly alter PLD lipase activity (Figure 15A). This result means that PARN overexpression decreased PLD2 gene expression independent of PARN’s deadenylase activity. However, since the PARN-H377A mutant still contains intact RNA-binding domains, it is possible that PARN could still bind mRNA targets and inhibit their translation/induce transcript degradation. When we induced overexpression of PLD2, we observed an upregulation in PARN gene expression that was abolished by overexpression of the lipase-inactive PLD2-K758R mutant (Figure 15B). Thus, the catalytic activity of PARN does not seem to be necessary for PLD2 regulation, but the catalytic activity of PLD2 is necessary for PARN regulation.

**Phosphatidic acid increased both PARN and PLD2 protein expression.**

As only catalytically active PLD2 had a positive effect on PARN expression (Figure 15B), we investigated what effect phosphatidic acid, PA (the product of the PLD reaction), might have on PARN. PA is a strong mitogen and regulator of gene transcription (Yoon et al., 2015). COS-7 cells were incubated with increasing concentrations of 1,2-dioleoyl-sn-3-phosphate (dioleoyl-PA) for either 20 min or 4 h. As shown in Figure 16A-B, both PLD2 and PARN protein expression increased concomitantly with PA concentration. Quantifications of protein bands are shown in Figure 16C-F. PLD2 protein expression increased during this 4 h incubation up to a peak at 100 nM dioleoyl-PA followed by a decrease at higher concentrations of dioleoyl-PA, while PARN protein increased throughout (Figure 16D,F). A similar effect was seen during a shorter incubation time (20 min) (Figure 16C,E). These results indicate there is a concentration dependent threshold to which the cells produce more protein in response to PA.
Figure 15. PLD2 catalytic activity is required to increase PARN gene expression, while both wild-type and the catalytically inactive PARN decrease PLD2 gene expression. (A) PLD2 gene expression in COS-7 treated with mock transfection alone or PLD2-WT or lipase-inactive PLD2-K758R mutant were each overexpressed. (B) PARN gene expression in COS-7 cells mock transfection alone or PARN-WT or deadenylase-inactive PARN-H377A mutant were each overexpressed and used to measure mRNA levels via qRT-PCR. Results are relative to Actin housekeeping gene. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05, significant increase between samples; #P<0.05, significant decrease between samples.
Figure 16. Exogenous dioleoyl-PA increases PARN and PLD2 protein expression.

COS-7 cells were incubated with increasing concentrations of exogenously added dioleoyl-PA for 20 min (A,C,E) or 4 h (B,D,F). PARN and PLD2 protein expression were assessed via Western Blot. Results from 20 min dioleoyl-PA treatment are shown in (A), while results from 4 h dioleoyl-PA treatment are shown in (B). PARN protein densitometry is graphically represented in (C), for 20 min dioleoyl-PA treatment, and in (D), for 4 h dioleoyl-PA treatment. PLD2 protein densitometry is graphically represented in (E), for 20 min dioleoyl-PA treatment, and in (F), for 4 h dioleoyl-PA treatment. Actin was used as the equal protein loading control. Western blot images are representative of n=3.
Exogenous addition of phosphatidic acid to cell cultures enhances PARN deadenylase activity, as measured in vitro.

We next investigated if PA affects the deadenylase activity of PARN. To accomplish this goal, we set up a robust and reliable in vitro PARN deadenylase assay to validate PARN enzymatic activity, as shown in Figure 17-19. In Figure 17, [\(^{32}\)P]-\(\gamma\)ATP-radiolabeled A\(_{15}\) RNA substrate was deadenylated by purified recombinant PARN protein, which has a different mobility in the denaturing gel than that of the boiled (inactive) purified recombinant PARN protein and the A\(_{15}\) substrate alone. Deadenylation was evidenced by an increased mobility of radiolabeled and degraded products (the smeared product) versus the input A\(_{15}\) and boiled PARN negative controls alone. Figure 18A shows that PARN protein but not recombinant, purified PAN2 protein (another closely related deadenylase) deadenylated the A\(_{15}\) substrate, which suggests the A\(_{15}\) substrate is specific for PARN and not for another deadenylase. Figure 18B depicts a Coomassie-stained gel that indicates the high purity of the recombinant, purified proteins used. PARN deadenylase activity was effectively silenced in COS-7 cells with siPARN RNA (Figure 18C) but not with siPAN2 RNA. This indicates that in our assay conditions, PAN2 did not contribute to the deadenylase activity found in cell lysates. A further control for these activity experiments is shown in Figure 19, whereby PARN activity in lysates from cells overexpressing wild-type PARN increased in a concentration-dependent manner when compared to overexpression of the deadenylase-inactive mutant PARN-H377A.

After thoroughly validating the PARN activity assay, we wanted to see if incubating COS-7 cells with dioleoyl-PA would not only increase PARN protein expression, but also correspondingly increase PARN activity. COS-7 cells incubated with 300 nM PA for 30 min and 30 nM PA for 4 h showed increased PARN activity compared to the negative control (A\(_{15}\) substrate alone, no lysate) and Mock (no PA treatment) (Figure 20). These results go hand-in-hand with the protein expression experiment where PA increased PARN expression. These data indicate PA positively affects PARN expression and activity in non-cancerous cells. This could be one mechanism by which COS-7 cells use to respond to upregulation of PLD2 in an attempt to bring cellular PLD2 levels back to normal through upregulation of PARN expression and activity. We
propose this mechanism is either not present in breast cancer cells or is somehow dysregulated, which allows the uncontrolled and sustained upregulation of PLD2 in these cells.

**PARN co-localizes with PA-containing vesicles in the cell and directly binds saturated PA species.**

We wanted to determine if the effects of PA on PARN expression and activity are through direct interaction between PARN protein and the phospholipid itself. No published studies thus far have investigated a relationship between PARN and any type of lipid. First, we wanted to determine if PARN protein localizes with PA. To accomplish this we incubated COS-7 cells for 30 min with 30 nM NBD-PA, a green fluorescently tagged PA similar in structure to the dioleoyl-PA used in all PA experiments thus far. We found that this NBD-PA formed distinct punctae and vesicular structures throughout the cells, but these structures were concentrated near the perinucleus (Figure 21, green panel). PARN was concentrated inside the nucleus, presumably in the nucleoli, but also highly concentrated in the same vesicle structures as the NBD-PA (Figure 21, red panel). When merged, this co-localization of PARN with the NBD-PA vesicles became much clearer (Figure 21, merged image).

Second, now that we have established that PARN protein comes into spatial contact with exogenously added PA, we wanted to determine if PARN physically binds phospholipids. PVDF membrane was spotted with equal concentrations of seven different phospholipids: 1,2-dioleoyl-sn-3-phosphate (DOPA), 1,2-diarachidonyl-sn-glycero-3-phosphate (AraPA), 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA), 1,2-dilauroyl-sn-glycero-3-phosphate (DLPA), 1-oleoyl-sn-glycero-2,3-cyclic-phosphate (Lyso-PA), 1,2-dioctanoyl-sn-glycero-3-phosphocholine (PC), and L-a-Phosphatidylinositol-4,5-bisphosphate (PIP2). This membrane was then incubated with purified PARN protein and probed with PARN antibody. If PARN protein bound a lipid, PARN antibody would also bind and allow us to visualize the spots. As shown in Figure 22A, PARN only bound DMPA, DLPA, and PC (spots 4, 5, and 7 respectively). PARN did not bind the dioleoyl-PA (DOPA), the PA that closely resembles that of endogenous PA produced by PLD action. Since this is the PA we have used in our PARN expression and activity
experiments, this indicates that the effects observed are due to some other downstream signaling and not direct binding of DOPA to PARN. Figure 22B shows a control blot spotted with solvent alone or DOPA and there were no further treatments with blocking buffer or antibodies. This blot was placed in a box to stain the lipid with iodine vapors. The DOPA spot was stained yellow showing that our lipids bound the paper.

A model of regulation between PLD2/PA and PARN.

A model representing the proposed regulation we have characterized between PLD2/PA and PARN is represented in Figure 23. The results of this study indicate that PA, the hydrolysis product of PLD action, enhanced the activation and synthesis of PARN protein. As illustrated in Figure 23A, when PLD2/PA levels increased in normal cells, like COS-7, this caused an increase in PARN gene and protein expression, as well as an increase in its deadenylase activity. This overall elevation in PARN levels in the cell then decreased PLD2 expression to basal levels. The mechanism by which this occurs still needs to be fully elucidated, as it is possible that PARN can degrade PLD2 mRNA directly and thus influence PLD2 expression levels, or by a more indirect mechanism where PARN degrades other mRNA that then affect PLD2 expression. As illustrated in Figure 23B, the large negative feedback of PARN on PLD expression appears attenuated in cancer cells, due to not only the inherently low basal PARN expression, but it also could be due to compromised degradation of target mRNAs or even a yet to be established regulatory pathway. Taken together these results further support the significant role of PLD2 and the importance of elucidating its regulation in breast cancer. This is also the first study to specifically focus on and attempt to discern a role for PARN in breast cancer and in the PLD regulation process.
Figure 17. Purified recombinant PARN protein and protein from COS-7 cell lysates deadenylate $A_{15}$ substrate. Validation study for in vitro PARN deadenylase activity assay. Radiolabeled $A_{15}$ RNA substrate was deadenylated by recombinant PARN with respect to the $A_{15}$-only control and inactivated (boiled) recombinant PARN protein. Deadenylation is evidenced by a greater mobility of radiolabeled spots and the appearance of smears versus the negative controls of inactivated PARN or $A_{15}$ alone.
Figure 18. The PARN activity assay is specific for PARN and not PAN2. Further validation of the specificity of the PARN deadenylase assay. (A) Recombinant PARN but not recombinant PAN2 is able to deadenylate A₁₅. (B) Coomassie-stained gel indicating the high purity of the recombinant, purified proteins used. (C) PARN deadenylase activity as measured in lysates from COS-7 cells that were silenced with 150 ng of either control RNA (siNeg), siPARN, or siPAN2.
Figure 19. The PARN-H377A mutant has decreased PARN activity compared to PARN-WT. PARN activity in COS-7 cells overexpressing either PARN-WT or -H77A proteins. Error bars are means ± s.e.m. The differences between means were assessed by t-test. #P<0.05, significant decrease between samples and control.
Figure 20. Exogenous addition of dioleoyl-PA increases PARN activity above mock levels. PARN activity of lysates prepared from COS-7 cells incubated with or without (Mock) the indicated concentrations of dioleoyl-PA for 30 min or 4 h. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05, significant increase between samples; #P<0.05, significant decrease between samples.
Figure 21. PARN protein co-localizes with NBD-PA containing vesicles. COS-7 cells were incubated for 30 min in 30 nM fluorescent PA (NBD-PA) and were then used for immunofluorescence microscopy using TRITC-conjugated PARN IgG antibodies. Image is representative of sextuplicate fields. Localization of the NBD-PA is in green (excitation=490 nm; emission=525 nm, using a FITC filter; top left panel), while localization of PARN is in red (excitation=557 nm; emission=576 nm, using a TRITC filter; bottom left panel). Nuclei were stained blue with DAPI and the images merged (right panel). White arrows point to areas of high co-localization (yellow), which correspond to vesicles localized perinuclear.
Figure 22. PARN binds saturated PA species but not dioleoyl-PA. (A) Results from a lipid-overlay assay incubated with purified recombinant PARN protein and probed with PARN antibody. Samples spotted were as follows: 1) solvent only; 2) DOPA; 3) AraPA; 4) DMPA; 5) DLPA; 6) Lyso-PA; 7) PC; and 8) PIP2. (B) Control blot spotted with solvent only or DOPA. Blot stained with iodine vapors to show that lipid binds the paper (yellow spot).
Figure 23. Proposed model for the interactions of PARN with PLD2 in noncancerous versus cancerous cells. (A) In non-cancerous cells, a positive and a negative feedback mechanism exists, whereby PLD2 and PA production initially upregulates PARN gene and protein expression. This PARN protein then decreases PLD2 expression by potentially degrading PLD2 mRNA directly, or by degrading other mRNA that affects PLD2 expression (a more indirect mechanism), bringing PLD2 levels back to a normal level. (B) Working model for the deregulation between PARN and PLD2 in cancerous cells. As in non-cancerous cells, PLD2 upregulates PARN. However, PARN cannot downregulate PLD2. Low basal PARN expression, compromised degradation of mRNA, or a yet to be established regulatory pathway may all contribute to higher PLD2 protein expression, which mediates the increased cell growth, proliferation, and invasion of these cells.
We have shown that both PLD1 and PLD2 are overexpressed in human breast cancer tissue as well as in human breast cancer cell lines, while PARN is downregulated in these same samples (Figure 6, 9-10). We also established that there is a regulatory relationship between PLD2 and PARN, not only in terms of gene and protein expression levels, but also in terms of enzymatic activities, although the exact mechanism behind this is still not understood. It is well known that PARN targets mRNA containing AU-rich elements (AREs) in their 3’ UTR. To see if PLD1 contained such a region, we searched the sequence of its 3’ UTR and found several stretches of AU-rich regions, one of which was quite long at the terminus of the 3’ UTR (approximately 783 nt long) (Figure 5, yellow). This region was also of particular interest to us because immediately preceding this region was a suspected miR-203 binding site (Figure 5, red). As shown previously in our lab, miR-203 is capable of targeting PLD2’s 3’ UTR and thus regulating its protein expression (Fite et al., 2016; Fite and Gomez-Cambronero, 2016). This same miR is also predicted to target PLD1. Taking these observations into account during our investigation into PARN regulation of PLD, we shifted our focus to PLD1 as we believe a direct mechanism for PARN regulation on this PLD does in fact exist. As the PLD2 3’ UTR contains miR targeting sites, miR-203 being one of them, it does not contain any significantly sized AREs. We thus set out to determine if PARN directly regulates PLD1 through interaction with the PLD1 3’ UTR and whether this was miR-203-dependent.

**PLD1 gene and protein expression increases in the absence of PARN.**

To determine if PARN is having an effect at all on PLD1 expression, we transiently silenced PARN in all four of our cell lines of interest: COS-7, HMEC, MCF-7, and MDA-MB-231. Figure 24 shows significant upregulation of PLD1 gene expression when PARN was knocked down in the COS-7 (Figure 24A-B), HMEC (Figure 24C-D) and MCF-7 (Figure 24E-F) cell lines, however this effect was not seen in the MDA-MB-231 breast cancer cell line even though PARN gene expression was significantly down-regulated (Figure 24G-H). This indicated that under normal conditions (COS-7 and HMEC) PARN keeps PLD1 mRNA levels under control. This phenomenon seems to persist in the pre-EMT low aggressive MCF-7 breast cancer cell
line, but it is not present to the same degree in the post-EMT highly aggressive MDA-MB-231 breast cancer cell line.

**PARN directly regulates the PLD1 3’ UTR.**

To confirm that PARN protein directly binds and regulates PLD1 expression via interaction with the PLD1 3’ UTR, we performed a luciferase reporter assay. As shown in Figure 25, if PARN regulates PLD1 3’UTR expression, then when PARN binds the PLD1 3’ UTR within the luciferase fusion mRNA, translation will be inhibited and less luciferase signal will be produced compared to the PARN silenced condition. Indeed, silencing of PARN in COS-7 cells resulted in an increase in PLD1 3’ UTR luciferase signal above that of the mock condition (Figure 26A), while the PLD1 3’ UTR luciferase signal greatly decreased from mock levels when PARN was overexpressed even at all concentrations tested (Figure 26B). This experiment was repeated with the PLD2 3’ UTR but yielded a much smaller effect (Figure 26C-D). These results together yield clear evidence that PARN strongly and directly regulates PLD1 expression through targeting of the PLD1 3’ UTR, but the same is not true for PARN regulation of the PLD2 3’ UTR. To further support PARN directly binding and regulating PLD1 mRNA, an RNA I.P. assay was performed with the cell lines of interest. After subjecting these cell lines to UV cross-linking to more stably bind protein complexes together, cells were collected and lysates prepared. PARN protein was pulled down using anti-PARN antibody bound to Protein G agarose beads and anti-IgG was used as a control. Any mRNA physically associated with PARN protein should also be pulled down (Figure 27, inset cartoon). After I.P., qRT-PCR was performed to measure the PLD1 mRNA potentially pulled down along with the PARN protein, which would indicate physical binding of PARN to that mRNA. COS-7, HMEC, and MCF-7 cell lines showed more PLD1 pulldown in the PARN I.P. samples compared to their corresponding IgG control (Figure 27). This was not observed in the MDA-MB-231 cell line, indicating that what PARN protein is present in these cells may have inhibited PLD1 mRNA binding capability in this aggressive breast cancer cell line, which might have contributed to the upregulation of PLD1 in this cell line. Next, we wanted to investigate the potential for this PLD1 regulation to not only involve PARN but also miR-203 either in a miR-dependent or -independent mechanism.
Figure 24. PLD1 gene expression increases in the absence of PARN in all cell lines except for MDA-MB-231. PLD1 and PARN gene expression for COS-7 (A-B), HMEC (C-D), MCF-7 (E-F), and MDA-MB-231 (G-H) cell lines measured by qRT-PCR relative to three housekeeping genes: TBP, GAPDH, and Actin. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05 and **P<0.01, significant increase between samples and controls.; ##P<0.01 and ####P<0.0001, significant decrease between samples and controls.
Figure 25. Scheme of PLD 3’ UTR luciferase assays. If PARN regulates the PLD 3’UTR expression, then PARN will bind the PLD 3’ UTR within the luciferase fusion mRNA. If this happens, then translation is inhibited and less luciferase signal is produced when compared to the PARN silenced condition.
Figure 26. PARN directly binds and regulates the PLD1 3’ UTR. (A) Luciferase assay results from COS-7 cells co-transfected with the PLD1 3’ UTR luciferase reporter plasmid and either siRNA negative control or siPARN. (B) Luciferase assay results from COS-7 cells co-transfected with the PLD1 3’ UTR luciferase reporter plasmid and either transfection reagents alone (Mock) or overexpressed PARN plasmid in the amounts indicated. (C) Luciferase assay results from COS-7 cells co-transfected with the PLD2 3’ UTR luciferase reporter plasmid and either siRNA negative control or siPARN. (D) Luciferase assay results from COS-7 cells co-transfected with the PLD2 3’ UTR luciferase reporter plasmid and either transfection reagents alone (Mock) or overexpressed PARN plasmid in the amounts indicated. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05 and **P<0.01, significant increase between samples and controls.; #P<0.05 and ####P<0.0001, significant decrease between samples and controls.
Figure 27. PARN protein is complexed with PLD1 mRNA in the normal, non-cancerous and low invasive breast cancer cell lines and much less so in the highly aggressive breast cancer cell line MDA-MB-231. PLD1 mRNA pulldown by PARN antibody compared to IgG control in COS-7, HMEC, MCF-7, MDA-MB-231 via RNA I.P. as measured by qRT-PCR. The companion Western Blot probed for PARN protein in the I.P. samples is shown below the graph.
MiR-203 directly regulates PLD1 gene and protein expression.

To confirm that miR-203 regulates PLD1 expression as predicted by TargetScan, we overexpressed miR-203 plasmid in MDA-MB-231 cells as we know from previous work in our lab that these cells have low endogenous levels of miR-203 (Fite et al., 2016; Fite and Gomez-Cambronero, 2016), but high levels of PLD1 (Figure 9-10). Figure 28A confirms the overexpression of miR-203 in the miR-203 transfected MDA-MB-231 cells. As predicted, miR-203 overexpression decreased both PLD1 gene expression (Figure 28B) and protein expression by over half (Figure 28C). When overexpressed with the PLD1 3’ UTR luciferase plasmid, miR-203 significantly decreased the luciferase signal compared to scramble mimic control indicating that miR-203 was able to directly bind to and regulate the PLD1 3’ UTR (Figure 28D). Taken together, these data suggest that miR-203 regulates PLD1 expression.

PARN regulation of the PLD1 3’ UTR is dependent on the presence of both the ARE and miR-203 binding site.

We wanted to determine whether PARN regulation of PLD1 is dependent on two features of the PLD1 3’ UTR: the ARE and the miR-203 targeting site. To accomplish this goal, we performed mutagenesis of the wild-type PLD1 3’ UTR LightSwitch luciferase reporter plasmid to create five new mutant plasmids. These mutants are pictorially represented in Figure 7. One mutant, ΔMIR, had the miR-203 targeting site completely removed. A second mutant, mutMIR, had the miR-203 targeting site mutated to disrupt miR-203 binding. The other three mutants are deletion mutants of portions of the ARE, where the last third of the ARE was removed (Δ1/3ARE), the last two-thirds of the ARE was removed (Δ2/3ARE), or the entire ARE up to the miR-203 targeting site was removed (ΔARE).

First we needed to confirm that miR-203 could no longer regulate the mutMIR and ΔMIR PLD1 3’ UTR mutants. As shown in Figure 29A, miR-203 overexpression no longer decreased luciferase signal in both the mutMIR and ΔMIR validating that these two mutants had abolished miR-203 binding. The red symbols indicate that these samples (mutMIR, Δ1/3ARE, Δ2/3ARE, and ΔARE) were significantly increased or decreased compared to the PLD1 WT 3’ UTR overexpressing miR-203. Next, we either silenced
PARN or overexpressed PARN in the presence of the mutant PLD1 3’ UTR luciferase constructs. When PARN was silenced, there was an increase in luciferase signal in both the WT and Δ2/3ARE samples, however, no significant increase in luciferase signal was observed in the mutMIR, ΔMIR, Δ1/3ARE, or ΔARE samples (Figure 30A). When PARN was overexpressed, there was a near abolishment in luciferase signal in the WT samples, whereas luciferase signal decreased to a significantly lesser extent in the Δ1/3ARE, Δ2/3ARE, and ΔARE samples. The red symbols indicate that these samples were significantly increased or decreased compared to the PLD1 WT 3’ UTR that were silenced or overexpressed PARN. Importantly, in corroboration with the PARN silencing results, no significant decrease in luciferase signal was observed in the mutMIR and ΔMIR samples indicating that PARN regulation of the PLD1 3’ UTR requires the presence of the intact wild-type miR-203 targeting site strongly suggesting this is a miR-203 dependent mechanism (Figure 30B).

Model for PARN regulation of PLD1 expression.

In normal breast cells, such as HMEC (Figure 31A), PARN and miR-203 expression levels are high compared to their levels in breast cancer cells. PARN can physically associate with Ago2 in the RISC and once the RISC is loaded with miR-203, a 3’ UTR targeted interaction with PLD1 mRNA and this complex occurs leading to PLD1 mRNA degradation. This regulation keeps PLD1 levels tightly under control and at a “normal” basal level. However, in the highly aggressive breast cancer cell line, such as MDA-MB-231 (Figure 31B), endogenous levels of miR-203 and PARN are much lower than in normal breast cells, while PLD1 expression is significantly upregulated. With little to no miR-203 and/or PARN within these cells, PLD1 protein translation proceeds in a more uncontrolled manner leading to and/or maintaining upregulated PLD1.
Figure 28. miR-203 overexpression in MDA-MB-231 breast cancer cell line decreases PLD1 gene and protein expression through direct binding of the PLD1 3’ UTR. 

(A) miR-203 gene expression in MDA-MB-231 cells control or miR-203 plasmid overexpressed as measured via qRT-PCR. 

(B) PLD1 gene expression in MDA-MB-231 cells control or miR-203 plasmid overexpressed as measured via qRT-PCR. P-value was close to achieving significance at P=0.057. 

(C) PLD1 protein expression in MDA-MB-231 cells control or miR-203 overexpressed. TBP is used as equal protein loading control. Densitometry values normalized to TBP and relative to the control are shown. Images are representative of n=3. 

(D) Luciferase assay results from COS-7 cells co-transfected with the PLD1 3’ UTR luciferase reporter plasmid and either scrambled mimic or miR-203 mimic. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05 and ***P<0.005, significant increase between samples and controls.
Figure 29. Mutation and/or deletion of the PLD1 3’ UTR miR-203 targeting site abolishes miR-203 regulation of the 3’ UTR. Luciferase assay results from COS-7 cells co-transfected with the PLD1 3’ UTR luciferase reporter plasmids and either scrambled mimic or miR-203 mimic. Error bars are means ± s.e.m. The differences between means were assessed by t-test. *P<0.05 and ***P<0.005, significant increase between samples; ##P<0.01, significant decrease between samples. Red symbols denote significant increase/decrease compared to PLD1 WT 3’ UTR overexpressing miR-203.
Figure 30. Mutations in the PLD1 3’ UTR significantly reduce PARN regulation of the 3’ UTR. (A) Luciferase assay results from COS-7 cells co-transfected with the PLD1 3’ UTR luciferase reporter plasmids and either siRNA negative control or siPARN. (B) Luciferase assay results from COS-7 cells mock transfected (PLD1 3’ UTR luciferase reporter plasmids alone) or co-transfected with the PLD1 3’ UTR luciferase reporter plasmids and PARN plasmid. Error bars are means ± s.e.m. The differences between means were assessed by t-test. **P<0.01, ***P<0.005, ****P<0.001, significant increase between samples. #P<0.05, ##P<0.01, and ###P<0.005, significant decrease between samples. Red symbols denote significant increase/decrease compared to PLD1 WT 3’ UTR siPARN (for panel A) or PARN overexpression (for panel B).
Figure 31. Proposed model for miR-203 dependent-PARN post-transcriptional regulation of PLD1. (A) In normal breast cells (HMEC), endogenous levels of miR-203 and PARN are relatively high compared to PLD1. PARN can physically associate with Ago2 in the RISC and once the RISC is loaded with miR-203, a 3’ UTR-targeted interaction with PLD1 mRNA and this complex occurs leading to PLD1 mRNA degradation. (B) In the highly aggressive breast cancer cell line MDA-MB-231, endogenous levels of miR-203 and PARN are much lower than in normal breast cells, while PLD1 expression is significantly upregulated. With little to no miR-203 and/or PARN within these cells, PLD1 protein translation occurs in a more uncontrolled manner leading to and/or maintaining upregulated PLD1.
IV. DISCUSSION

The relationship between phospholipases and RNases is interwoven in terms of cellular location and function. Previous studies have linked phospholipase C (PLC) to RNase activity, which serves as a basis for the possibility of other phospholipases being involved in RNase activities, such as PLD2/PA. It has been previously determined that PLD and ribonucleases co-localize in the vacuoles of plants (Matile, 1968). It is well established that PARN exists both in the cytoplasm and nucleus, whereas PLD2 has been shown to be in the plasma membrane and cytoplasm and its secondary messenger, PA, can be found throughout the cell to exert a variety of functions in cell signaling (Hondal et al., 1997; Kravchuk et al., 2001; Zini et al., 1989). Indeed, while immunofluorescence microscopy and co-IP techniques have not shown definitive evidence of PARN co-localizing or physically interacting with PLD itself (data not shown), our IF experiments have indicated that PARN can co-localize with PA inside the cell (Figure 21). Taken together, the research on PLC and current understanding of the subcellular localization of PLD2, PA and PARN support the notion that these molecules have ample opportunity to have a direct and/or indirect interaction with one another.

This work identifies that the phospholipid signaling enzyme PLD2 and its catalytic product, PA, are a novel route for modulating PARN expression and activity. We report that a possible inter-regulatory relationship exists, whereby PLD2 affected PARN gene and protein expression, as well as activity, and vice versa. This mechanism appears to be modulated differently in non-cancer versus cancer cells. The results shown here support a potentially important role for PA and PLD2 in PARN-mediated mRNA decay. It is known that PA can activate transcription factors such as PPARα, so it is possible that an increase in PA may activate transcription factors that could subsequently increase PARN transcription. Most critically, we also observed strong regulation of both PLD1 and PLD2 in non-cancer cells via PARN. While we were not able to elucidate the exact mechanism by which PARN regulates PLD2, we did find that PARN regulates PLD1 via interaction with the PLD1 3’ UTR. This interaction and regulation is dependent on the presence of the miR-203 targeting site nearest the 3’ terminus of the PLD1 3’ UTR, as well as strongly dependent on the presence of a large AU-rich element (ARE) directly
flanking this miR-203 site. The PARN-mediated regulation of both PLD1 and PLD2 appears to be impaired or nonexistent in highly aggressive breast cancer cells, such as MDA-MB-231, indicating a critical function for the interplay between PARN and both PLD isoforms.

The physiological relevance in the proposed regulation and interactions herein lie in the observed discrepancies between what occurs in normal or non-cancerous cell lines and what occurs in the highly aggressive MDA-MB-231 breast cancer cell line. By using normal/noncancerous and breast cancer cell lines that were mock-treated or that overexpressed PARN and/or PLD2, we defined the relationship between PARN and PLD2 (Figure 12-14). First and foremost, cancer cell lines have increased levels of PLD2 (Figure 9-10) and, therefore, higher levels of PA production (Foster, 2009; Foster et al., 2014). The product of PLD hydrolysis of membranous PC is almost exclusively monounsaturated PA (e.g. dioleoyl-PA), and as such, we used exogenously added dioleoyl-PA in our experiments. We believe that our results are evidence of a negative feedback mechanism in cells, whereby PLD2 overexpression and also PA positively affected PARN gene and protein expression, as well as activity, which in turn negatively affected PLD2 gene and protein expression and activity bringing PLD2 back down to normal mock level. This negative feedback mechanism between PARN and PLD2 was observed clearly in our normal cell line, COS-7, but was diminished in our breast cancer cell line, MCF-7, possibly due to an as-yet-unidentified regulator or pathway that leads to the attenuation of the large negative feedback of PARN on PLD2 expression. This also demonstrated that immortalization of COS-7 cells was not sufficient for explaining the effects seen. Cancer cells having low levels of PARN and/or mechanisms in place to inhibit PARN action on certain mRNA could potentially maintain a more active pool of mRNA that facilitate overexpression of proteins like PLD2, which are highly relevant to tumorigenesis, cell migration and metastasis in breast cancer.

Not only have we begun to characterize a complex regulatory relationship between PARN and PLD2, we identified and characterized a mechanism by which PARN regulates PLD1. While there are several papers supporting the existence of a miR-dependent mechanism in deadenylases such as CCR4-NOT and the PAN2-PAN3 complexes, only one paper exists that actually shows evidence of this phenomena with
PARN. The PLD1 3' UTR is AU-rich and therefore there are many regions by which PARN may interact with the mRNA. Here we chose a large ARE in the 3' terminus of the 3' UTR not only because of its size and proximity to the poly-A tail but also because of the miR-203 targeting site immediately preceding the region, all of which would yield the highest probability of the existence of an ARE and miR-dependent mechanism by which PARN regulates PLD1. While we confirmed the miR-targeting site mutant PLD1 3' UTR luciferase constructs were not regulated by miR-203, the Δ1/3ARE and Δ2/3ARE constructs actually increased in luciferase signal with the overexpression of miR-203. This means there was a decreased ability of miR-203 to bind these two specific constructs. Although further experimentation would be required to pinpoint the exact reason for this phenomena, two possible explanations are that miR-203 may require a certain length ARE to bind properly and/or these two constructs fold in such a way that excludes miR-203 from its binding site (Hon and Zhang, 2007). PARN regulation of the PLD1 3' UTR was abolished in the miR-203 targeting site mutants, however a variation in responses was seen with the three ARE mutants. Between PARN silencing and overexpression, it was clear that deleting the last third of the 3' UTR was not enough to abolish PARN regulation, while it did significantly decrease it. Near complete abrogation of PARN regulation required the deletion of the entire ARE. This is in stark contrast to the complete abolishment seen with the miR-203 targeting site mutants, indicating that PARN regulation of the PLD1 3' UTR is most dependent on the presence of the miR-203 targeting site and to at least the first two-thirds of the identified ARE. It is important to note that COS-7, HMEC, and MCF-7 cell lines have higher levels of miR-203 and PARN compared to the highly aggressive MDA-MB-231 breast cancer cell line. Because of this, the proposed miR-203/PARN regulatory mechanism to keep PLD1 levels in check would be mostly if not completely absent. We propose this mechanism to be at least partially responsible for the upregulated expression of PLD1 in the MDA-MB-231 breast cancer cell line although the reason for the low levels of miR and PARN in these cells is not yet known.

In conclusion, we report here a regulatory relationship between PARN and both PLD isoforms, which constitutes a new and possibly essential component in post-transcriptional regulation in higher eukaryotes, as well as in disease states such as cancer.
Further investigation is needed into determining how and why the interaction between these proteins becomes dysregulated in breast cancer to start developing new methods for re-establishing normal interaction and remedying or preventing the cancerous pathology.
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