PELP1 oncogenic functions involve CARM1 regulation

Monica Mann1,2, Valerie Cortez1,2 and Ratna Vadlamudi1,2,6

1Department of Cellular and Structural Biology and 2Department of Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, TX, USA and 3Cancer Therapy and Research Center, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

*To whom correspondence should be addressed. Division of Reproductive Research, Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, Mail Code 7836, San Antonio, TX 78229-3900, USA. Tel: +1-210-567-4930; Fax: +1-210-567-4958; Email: vadlamudi@uthscsa.edu

Estrogen receptor alpha (ERα) is implicated in the initiation and progression of breast cancer and its transcription depends on the modulation of epigenetic changes at target gene promoters via coregulators. There is a critical need to understand the molecular mechanism(s) by which deregulation of epigenetic changes occurs during breast cancer progression. The ERα coregulator PELP1 plays an important role in ERα signaling and is a proto-oncogene with aberrant expression in breast cancer. PELP1 interacts with histones and may be a reader of chromatin modifications. We profiled PELP1’s epigenetic interactome using a histone peptide array. Our results show that PELP1 recognizes histones modified by arginine and lysine dimethylation. PELP1 functionally interacts with the arginine methyltransferase CARM1 and their interaction is enhanced by ERα. PELP1–CARM1 interactions synergistically enhance ERα transactivation. Chromatin immunoprecipitation assays revealed that PELP1 alters histone H3 arginine methylation status at ERα target gene promoters. Pharmacological inhibition or small interfering RNA knockdown of CARM1 substantially reduced PELP1 oncogenic functions. The critical role of PELP1 status in modulating arginine methylation status was also observed through in vivo studies where PELP1 knockdown mediated decreased tumorigenesis correlated with decreased arginine dimethylation. Further, immunohistochemical analysis of human breast tumor tissues revealed co-overexpression of PELP1 and CARM1 in a subset of ERα-positive breast tumors. Our findings show PELP1 is a reader of histone arginine methyl modifications and deregulation promotes tumor proliferation via epigenetic alterations at ERα target promoters. Targeting these epigenetic alterations through inhibition of PELP1 and the arginine methyltransferases could be a promising cancer therapeutic.

Introduction

Breast cancer is the second leading cause of cancer-related death in women and about 70% of breast tumors are positive for estrogen receptor alpha (ERα) expression at diagnosis (1). Estrogen signaling pathways have a central role in regulating the growth and survival of breast tumor cells. Despite the numerous therapies developed for the treatment of ERα-positive breast cancer, there are still a significant number of deaths each year that necessitate the development of additional treatment strategies. A leading challenge is the resistance of cancer cells to hormonal therapy and understanding the mechanisms behind this resistance will provide valuable insight that could be used to predict therapy resistance and tailor therapy to individual patients (1). A possible mechanism for drug resistance could be the epigenetic regulation of genes in estrogen signaling (2). Estrogen signaling plays a critical role in breast tumorigenesis; however, important knowledge gaps remain about the role of post-translational modifications in the initiation and progression of breast cancer (2).

Estrogen stimulation induces several histone modifications at ERα target gene promoters, including acetylation, phosphorylation and methylation (2). The mechanism by which ERα targets and coordinates the activities of histone modifying enzymes is poorly understood; therefore, studying the epigenetic regulation is critical to understanding ERα function in breast cancer and ultimately the development of better treatment (3). Transcription of ERα is regulated by several coactivators including PELP1 (proline-, glutamic acid- and leucine-rich protein 1) and the secondary coactivator CARM1 (coactivator-associated arginine methyltransferase 1) (4). Dimethylation of arginine residues 17 and 26 within histone H3 has been linked to active transcription (5). Proteins arginine methyltransferases (PRMTs) are recruited to promoters and other regulatory units to control gene expression by the methylation of histones (6). CARM1/PRMT4 is a transcriptional coactivator with dysregulated expression in vivo, and it is necessary for the estrogen-induced proliferation of breast cancer cells via E2F1 and its target genes and is involved in differentiation (7).

PELP1 plays an important role in ERα signaling and functions as a proto-oncogene with aberrant expression in hormone-related cancers (8). Increased PELP1 expression is correlated with larger tumor size, higher histological grade and shorter breast cancer-specific survival (8). PELP1 acts as a scaffolding protein involved in estrogen signaling, interacts with ERα through LXXLL motifs in its N-terminal region and participates in ERα transcription function (9). PELP1 is involved in oncosogenesis through its interaction with histones, the acetytransferases CBP and p300, deacetylases and the demethylase KDM1/LSD1 (10). PELP1 signaling is implicated in the development of hormonal therapy resistance (11). The molecular mechanisms by which PELP1 promotes oncosogenesis remain elusive; however, our studies show that PELP1 interacts with histones and is a novel reader of chromatin modifications (12,13). The specificity of PELP1 to various histone modifications and whether PELP1 interacts with arginine-modified histones or arginine methylases has now been elucidated.

In this study, we examined the specificity of PELP1 in recognizing histone modifications using modified histone peptide arrays and determined the role of arginine methylation in PELP1-driven oncosogenesis. Our results show that PELP1 recognizes arginine methyl modified histones. Further, our data demonstrate that PELP1 forms a complex with CARM1 to regulate its methyltransferase activity. Pharmacological inhibition of CARM1 or knockdown by small interfering RNA (siRNA) substantially reduces PELP1 oncogenic functions in vitro. Together, these results suggest that the PELP1–CARM1 axis determines the arginine methylation code at ERα target genes leading to breast cancer progression.

Materials and methods

Cell culture and chemicals

The ZR75 and MCF7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). ZRGST, ZRPELP1GST, MCF7GFP and MCF7PELP1GFP stable cell lines were described earlier (14). All of the model cells were passaged in the user’s laboratory for fewer than 6 months after receipt or resuscitation. Antibodies used were purchased from various companies: H3Rme2a (cat# 39709) and CARM1 (cat# 39251) from Active Motif (Carlsbad, CA); H3R17me2a from Millipore (cat# 07-215, Billerica, MA); glutathione-S-transferase (GST) from Cell Signaling Technology (cat# 2624, Beverly, MA) and PELP1 from Bethyl Laboratories (Montgomery, TX). CARM1 siRNA was purchased from Dharmacon (cat# L-004130-00, Lafayette, CO) and transfectected using lipofectamine as described previously.
PELP1 regulates arginine methylation

Arginine N-methyltransferase inhibitor 1 (AMI-1) was purchased from Enzo (cat# ALX-270-440, New York, NY).

Histone peptide array

The modified histone peptide arrays were purchased from Active Motif (cat# 13008) and analyzed per manufacturer’s protocol. Briefly, the array was blocked in 5% non-fat dry milk/tris-buffered saline and tween 20 (NFDM/TTBS) on a shaker overnight at 4°C. It was then washed three times with TTBS buffer and incubated with purified protein either from ZRPELP1GST cells or from full-length bacterial PELP1-GST or bacterial PELP1-GST 800–960 in interaction buffer (100 mM KCl, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.9, 1 mM ethylenediaminetetraacetic acid, 0.1 mM dithiothreitol, 10% glycerol) overnight on a shaker at 4°C. The array was then washed five times with TTBS and incubated with primary antibody anti-PELP1 (1:5000, Bethyl Laboratories, cat# A300-180A) or anti-GST (1:1000, Cell Signaling Technology, cat# 624). To generate GST-PELP1 expression vector, the codons of the full-length human PELP1 were optimized for bacterial expression, PELP1 gene was synthesized using GenScript services, subcloned into the PGEX-6P1-GST vector and PELP1 protein was induced by the addition of isopropyl-thio-β-d-galactoside for 12 h at 27°C. GST-PELP1 purification was achieved using GST beads. The analysis of the array was done using the software provided by Active Motif. The slide has two duplicate arrays that are analyzed together using the software. The software performs analysis by specificity for each modification that is the ratio of the average intensity of all spots containing the mark divided by the average intensity of all spots not containing the mark. The background level was defined to remove non-specific signals and a graph of the distribution errors displays the errors of the intensities between the right and left spot, normalized to the maximum intensity according to the Active Motif Analysis Software.

Cell extracts, western blots and immunoprecipitation

Cell lysates for western blot and immunoprecipitation were prepared as described previously (13).

Cell viability, soft agar assay and migration assays

Proliferation assays were done as described previously (14). The soft agar assays were performed as described previously (15). Migration assays were done using modified Boyden chamber assays as described previously (16).

Peptide and GST pull-down assays

Peptide pull-down assays were done according to established protocol (17). Biotin peptides were purchased from Anaspec (cat# 65233-025, 65234-025, 65351, 65261, 64634-025, 64630, 64608-025, 61702) and avidin agarose from Thermo Scientific (cat# 20219). Pull-down assays were performed using bacterial PELP1-GST deletions described previously (13).

Histone methyltransferase assay

Recombinant CARM1 (cat# 31347, Active Motif) and histone H3 (cat# 14-411, Millipore) were used for the histone methyltransferase assay. The manufacturer’s protocol was followed: histone H3, S-adenosyl-l-methionine, CARM1 and purified bacterial full-length PELP1 were added in methyltransferase buffer (5×: 100 mM Tris–HCl, pH 8.0, 1 M NaCl, 2 mM ethylenediaminetetraacetic acid) and incubated for 2 h at 30°C. The reaction was stopped by the addition of Laemmli reducing sample buffer and run on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel.

Reporter gene assays and plasmids

Reporter gene assays were performed using the ERE-luciferase plasmid as described previously with CARM1 (Invitrogen) and ERE-luciferase plasmids.
After 72 h in 2.5% Dextran:charcoal stripped serum (DCC)-RPMI, the cells were treated with AMI-1 (10 μM) for 3 h and with either vehicle or estradiol (10⁻³ M) for 12 h. The cells were lysed with passive lysis buffer and luciferase activity was measured by the Luciferase Assay System (cat# E1501, Promega, Madison, WI). Each transfection was carried out in 6-well plates in triplicate and normalized with either β-gal activity or the total protein concentration.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (12). Sequential ChIP was performed using the Re-ChIP-IT Magnetic Chromatin Re-Immunoprecipitation Kit (cat# 53016, Active Motif) according to the manufacturer’s protocol with anti-PELP1, anti-CARM1 and anti-immunoglobulin G (IgG) antibodies. Primer sequences were GREGIC—forward: TTGTGTGACTTCCTGGGAGCA; reverse: CACACCGCCAGGCGTAAAG and TFFI—forward: GCTTAGGCTAGCAGGAATG; reverse: CAGGCTTCCTACTCATCTCGAG.

**Immunohistochemistry**

Tissues from previously established xenografts were used for immunohistochemical analysis (18). In brief, Nahu mice were inoculated with MCF7-PELP1 cells and treated with either control siRNA or PELP1 siRNA liposomes. Immunohistochemistry (IHC) was done according to previously established protocol with anti-PELP1 (1:200, cat# IHC-00013, Bethyl Laboratories), anti-CARM1 (1:50, cat# IHC-00045-1, Bethyl Laboratories) and anti-ER (1:50, cat# SC-7207, Santa Cruz Biotechnology) antibodies. Arrays were scored for the primary antibody with control rabbit IgG. The sections were scored by two independent evaluators blinded to the patient’s clinical status.

**Results**

**PELP1 uniquely recognizes several histone modifications**

There are several post-translational histone modifications involved in cancer including acetylation, phosphorylation, citrullination and methylation. Reader proteins that recognize these modifications facilitate modulation of genes and their resulting biological actions (6). We have shown previously that PELP1 acts as a module for recognition of histones through its carboxyl-terminal glutamic acid-rich region (13). To explore the PELP1 epigenetic interactome, we used a histone peptide array containing 384 combinations of histone modifications. We hybridized purified PELP1 protein from three sources including full-length bacterial PEPLL1, the histone binding region (800–960 amino acids) of bacterial PELP1 and full-length PELP1 isolated from ZR75-PELP1 breast cancer cells (Figure 1a; Supplementary Figure S1d and Table S1, available at Carcinogenesis Online) and analyzed recognition by western blot analysis. The GST control array had no signal (Supplementary Figure S1b, available at Carcinogenesis Online) and all of the arrays according to the Allred Score (19). Briefly, the staining intensity was scored on a scale between zero and three and the proportion of positive stained cells was rated as one between 0 and 1% positive, two between 1 and 10%, three between 10 and 33%, four between 33 and 66%, and five between 66 and 100%. The preparation of negative controls was accomplished by replacing the primary antibody with control rabbit IgG. The sections were scored by two independent evaluators blinded to the patient’s clinical status.

**Fig. 2.** PELP1 functionally interacts with CARM1. (a) An in vivo coimmunoprecipitation of PELP1 from nuclear lysate of ZR75 breast cancer cells is shown with immunoprecipitation of IgG as the control. The cells were estrogen starved in 2.5% DCC-RPMI for 72 h and treated with estradiol (10⁻³ M) for 12 h before nuclear lysates were prepared. PELP1 and CARM1 expression is shown by western analysis (upper panel). The reverse coimmunoprecipitation is also shown by immunoprecipitation of CARM1 from nuclear lysate of ZR75 breast cancer cells (bottom panel). (b) An in vitro PELP1-GST deletion binding assay was performed to map binding of CARM1. Recombinant CARM1 was incubated with PELP1-GST deletions bound to glutathione agarose with GST bound to glutathione agarose as the control. CARM1 expression is shown by western analysis with total CARM1 input. The reverse coimmunoprecipitation is also shown by immunoprecipitation of CARM1 from nuclear lysate of ZR75 breast cancer cells (bottom panel). (c) An in vitro binding assay with recombinant CARM1 and full-length PELP1 bound to glutathione agarose was performed in the presence or absence of purified ERα protein with bacterial GST bound to glutathione agarose as the control. CARM1 expression is shown by western analysis with total CARM1 input. Purity of PELP1-GST deletion proteins is shown in Supplementary Figure S1b, available at Carcinogenesis Online. (d) An in vitro binding assay with recombinant CARM1 and full-length PELP1 bound to glutathione agarose was performed in the presence or absence of purified ERα protein with bacterial GST bound to glutathione agarose as the control. Western analysis of PELP1, CARM1 and ERα expression is shown with total CARM1 input. (d) A reporter gene assay was performed using the ERE-luciferase plasmid. ZR75 or ZR75-PELP1KD cells were plated in 24-well plates and transfected with both ERE-luciferase and CARM1 plasmids. After 72 h in 2.5% DCC-RPMI, the cells were treated with AMI-1 (10 μM) for 3 h and with either vehicle or estradiol (10⁻³ M) for 12 h. The cells were lysed with passive lysis buffer and luciferase activity was measured by Luciferase Assay System (cat# E1501, Promega) in triplicates and analyzed by analysis of variance (ANOVA) using Prism Software (*P < 0.05, **P < 0.001). (e) An in vitro histone methyltransferase assay was performed with histone H3, recombinant CARM1 and purified bacterial PELP1. Western analysis of CARM1, PELP1, H3R17me2a and H3R26me2a is shown with total histone H3 as the control.
had a mean error range of the internal duplicates between 0 and 5%, indicating that PELP1 binding to the modifications is reproducible (Supplementary Figure S1a, available at Carcinogenesis Online) (20). Our results show that PELP1 recognizes histone H3 modified by arginine dimethylation, arginine citrullination and lysine dimethylation (Figure 1b). These results also validate previous findings that PELP1 reads histone H3 lysine dimethylation through its interaction with the histone lysine demethylase KDM1/LSD1 (13). PELP1 did not recognize modifications on histone H2 and H4 peptides, suggesting that PELP1 recognition of modifications depends on the sequence present in histones tails. Further, the glutamic acid-rich domain (PELP1-GST 800–960) also recognized dimethyl modified histones suggesting the Glu-rich domain of PELP1 is involved in the recognition. However, PELP1-GST 800–960 showed reduced specificity for some modifications such as H3R17citr compared with full-length PELP1, suggesting sequences surrounding the Glu region in PELP1 may further contribute to its affinity (Supplementary Figure S1c, available at Carcinogenesis Online). We also found that phosphorylation of residues adjacent to a methyl modification inhibits the ability of PELP1 to recognize histone methylation (Figure 1a). We chose to further investigate the novel finding of PELP1 recognition of histone arginine dimethylation, specifically H3R17me2a and H3R26me2a. The results from the histone peptide array were validated by an in vitro peptide pull-down assay (Figure 1c) showing that PELP1 recognizes H3R17me2a and H3R26me2a with strong affinity and other modifications such as H3R2me2 with less affinity (Supplementary Figure S1e, available at Carcinogenesis Online). Further, peptide pull-down assays with nuclear lysates of MCF7 (Figure 1d) and ZR75 (data not shown) cells also validated PELP1 recognition of H3R17me2a and H3R26me2a.

**PELP1 functionally interacts with CARM1**

The enzyme that catalyzes H3R17me2a and H3R26me2a modifications is the PRMT4 also known as CARM1 (21). Since PELP1 recognizes these modifications, we examined whether PELP1 interacts with CARM1. Initially, the complex formation of PELP1 and CARM1 was revealed through coimmunoprecipitation of PELP1 or CARM1 from nuclear lysates of both ZR75 (Figure 2a) and MCF7 breast cancer cells (data not shown) treated with estrogen. The coimmunoprecipitation results revealed that PELP1 and CARM1 interactions occur in breast cancer cells in an estrogen-dependent manner (Figure 2a). To map the region of PELP1 that interacts with CARM1, we performed an in vitro binding assay using various PELP1 deletions expressed as GST fusion proteins in *Escherichia coli* (Supplementary Figure S1d, available at Carcinogenesis Online) and found that CARM1 binds to the 400–600
amino acid region of PELP1 (Figure 2b). To determine if ERα is required for the complex to form, we then performed an in vitro binding assay with purified PELP1 and recombinant CARM1 in the presence or absence of ERα. We discovered that PELP1 and CARM1 form a weak complex; however, their interaction is further enhanced when ERα is present in the reaction (Figure 2c). To determine if PELP1 and CARM1 functionally interact, we performed ERE-luciferase reporter gene assays. The results from these experiments showed that PELP1 and CARM1 synergistically enhance ERα transactivation (Figure 2d) and knockdown of PELP1 significantly affects CARM1 transactivation functions. Earlier studies suggested that PELP1 modulated the enzymatic activities of chromatin modifying enzymes (13); therefore, we examined if PELP1 status affects CARM1’s methyltransferase activity using an in vitro methyltransferase assay. Our results revealed that the addition of purified PELP1 protein enhances CARM1’s methyltransferase activity shown by an increase in H3R17 dimethylation with increasing amounts of PELP1 (Figure 2c). Together, these results suggest that PELP1 functionally interacts with CARM1 and that PELP1 is needed for the optimal transcriptional activity of CARM1.

**PELP1–CARM1 interactions play a critical role in arginine dimethylation at ERα target genes**

Since both PELP1 and CARM1 function as coactivators of ERα, we performed a sequential ChIP using ZR75 cells treated with estradiol to determine if the two are corecruited to the same target genes. The sequential ChIP showed that PELP1 and CARM1 are corecruited to the estrogen responsive gene **GREB1C** (Figure 3a). Similar results were obtained with another ERα target gene **TFF1** (data not shown). ChIP assays revealed that CARM1 recruitment to the estrogen responsive promoter **GREB1C** is decreased when PELP1 expression is knocked down (Figure 3b). We next looked at global histone methylation levels in cells through histone isolation from ZR75 wild-type, PELP1 overexpressing ZR75-PELP1 cells and PELP1 knockdown cells. Both H3R17me2a and H326me2a levels positively correlated with PELP1 expression (Figure 3c). Further, ChIP assays of ZR75 wild-type or PELP1 knockdown cells revealed that PELP1 has the potential to alter histone H3 arginine dimethylation status at ERα target gene promoters. When both PELP1 and CARM1 are present, there is significant enrichment of both H3R17me2a and H326me2a modifications at estrogen responsive genes. However, when PELP1 expression is knocked down, the enrichment of both histone modifications is substantially decreased at **GREB1C** (Figure 3d and e).

**Inhibition of CARM1 decreases PELP1 oncogenic activity**

PELP1 has been shown to have several oncogenic properties including causing cellular transformation, metastasis and tumorigenesis (8). To test whether CARM1 is required for PELP1’s oncogenic properties, we performed proliferation, colony formation, migration and soft agar assays with CARM1 knockdown by

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**Fig. 4.** CARM1 required for PELP1 oncogenic functions. (a) Cell proliferation assay was performed using ZR75vec, ZR-PELP1 and ZR-PELP1KD cells. Cells were plated in a 96-well plate and after 72 h in 5% DCC, the cells were treated with estradiol (10⁻⁷ M) and with either vehicle or AMI-1 (0.5, 10 and 20 μM) every 48 h for 7 days. Viability was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide absorbance at 595 nm and triplicates were analyzed by t-test using Prism Software (**P < 0.05**). (b) Soft agar anchorage independence assays were performed using ZRvec and ZR-PELP1 cells. Cells were plated in the top agar layer in 30 mm culture plates and treated with either vehicle or AMI-1 (10 μM) every 72 h for 12 days. Total number of colonies were counted and analyzed by t-test using Prism Software (***P < 0.0001, ****P < 0.00001). (c) Colony formation assays were performed with MCF7vec, MCF7-PELP1, ZR75vec and ZR-PELP1 cells. Cells were plated in 6-well plates and treated with either vehicle or AMI-1 every 48 h for 12 days and colonies were stained with crystal violet. Colonies were counted and analyzed by ANOVA using Prism Software; all assays were performed in triplicate (*P < 0.05, **P < 0.001). (d) Cell migration assays were performed in duplicate using the QCM Colorimetric Cell Migration Assay Kit with MCF7vec and MCF7-PELP1 cells transfected with either siControl or siCARM1. Transfected cells were plated in the transwells with NIH3T3 conditioned media in the bottom well. After 18 h, the migrated cells were stained and absorbance measured at 595 nm. Analysis was done by ANOVA using Prism Software (***P < 0.001).
siRNA or pharmacological inhibition of CARM1 methyltransferase activity by AMI-1 (Supplementary Figure S2a and c, available at Carcinogenesis Online). Proliferation assays of ZR75 and MCF7 wild-type, PELP1 overexpressing and knockdown cells revealed that both CARM1 knockdown and inhibition of activity by AMI-1 decreased the PELP1-mediated proliferation (Figure 4a and Supplementary Figure S2b and d). The anchorage independence of ZR75-PELP1 overexpressing cells is also decreased by AMI-1 treatment (Figure 4b). Colony formation assays revealed that both AMI-1 treatment and siCARM1 cause a decrease in PELP1-mediated clonogenic potential (Figure 4c and Supplementary Figure S3a, available at Carcinogenesis Online). Boyden chamber migration assays with ZR75 and MCF7 wild-type and PELP1 overexpressing cells showed that treatment with both AMI-1 and CARM1 siRNA knockdown cause a decrease of PELP1-mediated migratory ability (Figure 4d and Supplementary Figure S3b, available at Carcinogenesis Online). Collectively, these results suggest that CARM1 plays an important role in mediating PELP1’s oncogenic functions.

PELP1 knockdown reduces histone H3 arginine methylation in xenograft tumors

Previous studies have shown that xenografts of MCF7-PELP1 cells in Nu/nu mice cause tumor formation without estrogen supplementation and that PELP1 knockdown by PELP1 siRNA liposomes causes a significant decrease in tumor growth and proliferation (18). Immunohistochemistry analysis of the PELP1 siRNA-treated tumors showed an 85% decrease in PELP1 expression (18). To determine if global histone arginine dimethylation at R17 or R26 is affected by PELP1 knockdown in xenografts, we performed immunohistochemistry on paraffin embedded tissues from previously established xenografts. We found that both of the histone modifications are substantially decreased in the PELP1 siRNA-treated tumors compared with the control siRNA-treated tumors (Figure 5a and b). We also found that PELP1 siRNA-treated tumors have a decrease in CARM1 expression (Figure 5a and b). Collectively, these findings suggest that PELP1 plays an important role in the modulation of CARM1 activity in vivo.

CARM1 expression is deregulated in breast tumors and correlates with PELP1 expression

PELP1 expression is commonly deregulated during breast cancer progression (22). Our studies indicate that CARM1 interacts with PELP1 and plays a critical role in PELP1’s oncogenic functions; we examined if CARM1 expression is deregulated during breast cancer progression and whether CARM1 expression correlates with PELP1 expression. IHC analysis of human breast cancer tissue microarrays showed increased expression of CARM1 in metastatic tumors compared with weak expression in normal breast tissue (Figure 6a and b). Statistical analysis revealed that CARM1 expression positively correlates with the level of PELP1 expression in a subset of ERα-positive invasive breast cancer tumors (Figure 6c). Collectively, these results suggest that deregulation of CARM1 expression occurs in breast cancer progression and that co-overexpression of PELP1 and CARM1 may occur in a subset of tumors.

Discussion

ERα plays a critical role in many aspects of breast tumorigenesis; however, important knowledge gaps remain about the role of post-translational modifications of ERα in the initiation and progression of breast cancer (2). There is a critical need to develop better therapies for breast cancer treatment and enhanced understanding of the mechanisms behind tumor progression and metastasis will aide in the development of novel therapeutics. PELP1 is a proto-oncogene that functions as an ERα coregulator, is highly expressed in breast cancer and has been shown to be involved in breast tumorigenesis and metastasis (15). In this study, we have identified PELP1 as a reader of histone arginine methyl modifications that are catalyzed by the enzyme CARM1 including histone H3 arginine 17 dimethylation as well as histone H3 arginine 26 dimethylation. PELP1 not only recognized these modifications but also worked synergistically with CARM1 in promoting estrogen activation and affected CARM1’s methyltransferase activity as well as its recruitment to estrogen responsive genes.

Recent genomics studies indicate that the balance between many of the histone marks is altered in cancer causing dysregulated states of gene transcription (2). The combination of histone modifications forms a ‘histone code’ that is read by proteins such as PELP1 and

Fig. 5. PELP1 knockdown affects arginine methylation in vivo. (a) Immunohistochemistry was performed on tissues from previously established protocol with MCF7-PELP1 xenografts in Nu/nu mice treated with control siRNA or PELP1 siRNA liposomes. Tissues were probed with anti-CARM1, anti-H3R17me2a and anti-H3R26me2a antibodies. Scale bars are 100 μm. (b) Quantification was done using ImageJ software with 10 tissue sections imaged per group at x20 magnification as percentage positive cells/total cells. Analysis was done by t-test using Prism Software (** P < 0.00001).
leads to distinct downstream events. Through such processes, histones are believed to function like a master ‘on/off’ switch to determine whether particular genes are active or inactive. Our studies using modified histone peptide arrays that contain 384 combinations of modifications revealed that PELP1 has the unique ability to recognize dimethyl modified histone H3 tails but not modified histone H2 or H4 tails. Further, a combination of arginine and lysine methylation on the histone H3 tail further increased PELP1’s affinity, whereas phosphorylation significantly reduced its affinity to these sites. These results suggest that physiological signals such as hormones and growth factors that promote phosphorylation pathways may indirectly alter epigenetic regulation by preventing recognition of certain epigenetic marks by readers such as PELP1. Since PELP1 functions as a coactivator of a number of nuclear receptors, PELP1’s ability to recognize histone methyl marks will have wide implications and may play a role in the activation of unique pathways when it is dysregulated.

CARM1 was originally identified as a coactivator of steroid receptors (21) including ERα and hormonal stimulation induces the cyclic recruitment of CARM1 to ERα target genes (23). CARM1 is also shown to play an essential role in estrogen-induced cell cycle progression in MCF7 cells via its cross talk with E2F1 (24). Some evidence also suggests that CARM1 may be involved in ER Swiss-mediated differentiation (25). Our results demonstrated that PELP1 has the potential

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**Fig. 6.** Coexpression of PELP1 and CARM1 in breast tumor tissues. (a) Immunohistochemical analysis of breast cancer progression tissue arrays (Biomax US) was performed with anti-PELP1 and anti-CARM1 antibodies. Analysis was done independently using the Allred Score by two analysts. Scale bars are 100 μm. (b) Quantitation of total IHC score for CARM1 in each tumor type; bars, SEM. Paired two-sample t-tests were used for analysis: 32 metastatic carcinoma, 68 invasive carcinoma, 22 each lobular carcinoma and intraductal carcinoma, 4 each of squamous cell carcinoma and lobular carcinoma in situ, 8 fibroadenoma, 16 each of hyperplasia and inflammation, 10 adjacent normal tissue and 6 normal tissue (*P < 0.05, **P < 0.01, ****P < 0.0001). (c) Coexpression analysis of PELP1 and CARM1 using ERα-positive invasive carcinoma samples (n = 23). Representative ERα-negative and ERα-positive IHC images are shown in lower panel. Pearson correlation coefficient was determined using Prism Software. Pearson correlation coefficient = 0.51, P = 0.007.
to modulate CARM1 function and activity. Further, in vivo PELP1 knockdown studies demonstrated that PELP1 is required for the optimal expression and recruitment of CARM1 to ERα target genes. It is possible that PELP1 regulation of CARM1 may require additional factors such as ERα. Since both PELP1 and CARM1 promote cell cycle progression by modulating E2F1 target genes, their interactions may also have implications in estrogen-mediated cell cycle progression by promoting optimal arginine histone methyl modifications (14,24). Since PELP1 is a recently identified proto-oncogene, a complete list of PELP1 target genes is not currently available. To clearly understand the impact of the PELP1–CARM1 axis, future studies are needed to determine the epigenetic target genes of PELP1 and CARM1. We will address this in our ongoing studies using genomic approaches such as ChIP-sequencing, RNA-sequencing and pathway analysis.

Emerging evidence also suggests that PELP1 is involved in oncogenesis through its interaction with histones, acetyltransferases CBP and p300, deacetylases and the demethylase KDM1 (9,13). Further, PELP1 may epigenetically regulate breast cancer progression and the development of hormonal therapy resistance (18). Studying these epigenetic interactions may elucidate the mechanism by which PELP1 promotes oncogenesis. Our results suggest that the interaction of PELP1 and CARM1 plays a critical role in modulating epigenetic changes during breast cancer progression by promoting arginine methylation at ERα target genes. Since PELP1 functions as a scaffolding protein, its selectivity to dimethyl modified arginine and lysine residues in histone H3 tails may provide a unique opportunity for PELP1 to assemble optimal histone modifying complexes to the specific regions in chromatin. Even though we have identified the binding domains of PELP1 that facilitate its interactions with modified histones and CARM1, we need to further clarify the mechanism for PELP1’s unique specificity. Future biochemical studies are clearly needed and will be pursued in our ongoing investigations.

PELP1 is a proto-oncogene that is overexpressed in several hormonal cancers including breast, prostate and ovarian cancers (8). Knockdown of CARM1 expression by siRNA or pharmacological inhibition of its activity resulted in inhibition of PELP1’s oncogenic properties including cellular proliferation, migration, anchorage independent and clonogenic potential. PELP1 siRNA-treated tumors had a decrease in arginine demethylation and CARM1 expression that could be explained by the fact that CARM1 is an ERα target and decrease of PELP1 results in a decrease of ERα signaling activation. Also, the expression of PELP1 and CARM1 correlated during breast cancer progression with high expression in ERα-positive invasive tumors. These results were also corroborated by findings from a recent study that found a positive association between the expression of PELP1 and CARM1 (22). These findings further suggest that both of the proteins work synergistically to promote tumorigenesis and metastasis. Therefore, regulating the epigenetic alterations at ERα target gene promoters, which occur during breast cancer progression, could be achieved by targeting the PELP1–CARM1 axis making it a promising cancer therapeutic.

In summary, our study demonstrates for the first time that PELP1 functions as a reader of arginine methyl modifications and plays a vital role in estrogen-mediated arginine methyl modifications of histone tails. Furthermore, we provided evidence that PELP1 functionally interacts with CARM1 and modulates its coactivator functions. Inhibition of CARM1 expression or function substantially reduces PELP1’s oncogenic functions. These findings suggest that deregulation of the PELP1–CARM1 axis may have implications on breast cancer progression via epigenetic alterations at ERα target promoters and that the PELP1–CARM1 axis may represent a promising cancer therapeutic.

Supplementary material
Supplementary Figures S1–S3 and Table S1 can be found at http://carcin.oxfordjournals.org/

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References

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