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Cyclin-Dependent Kinase–Mediated Phosphorylation Plays a Critical Role in the Oncogenic Functions of PELP1

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Abstract

Estrogen receptor (ER) signaling plays an important role in breast cancer progression, and ER functions are influenced by coregulatory proteins. PELP1 (proline-, glutamic acid–, and leucine-rich protein 1) is a nuclear receptor coregulator that plays an important role in ER signaling. Its expression is deregulated in hormonal cancers. We identified PELP1 as a novel cyclin-dependent kinase (CDK) substrate. Using site-directed mutagenesis and *in vitro* kinase assays, we identified Ser^{477} and Ser^{991} of PELP1 as CDK phosphorylation sites. Using the PELP1 Ser^{991} phospho-specific antibody, we show that PELP1 is hyperphosphorylated during cell cycle progression. Model cells stably expressing the PELP1 mutant that lack CDK sites had defects in estradiol (E2)–mediated cell cycle progression and significantly affected PELP1-mediated oncogenic functions *in vivo*. Mechanistic studies showed that PELP1 modulates transcription factor E2F1 transactivation functions, that PELP1 is recruited to pRb/E2F target genes, and that PELP1 facilitates ER signaling cross talk with cell cycle machinery. We conclude that PELP1 is a novel substrate of interphase CDKs and that its phosphorylation is important for the proper function of PELP1 in modulating hormone-driven cell cycle progression and also for optimal E2F transactivation function. Because the expression of both PELP1 and CDKs is deregulated in breast tumors, CDK-PELP1 interactions will have implications in breast cancer progression. *Cancer Res; 70(18); 7166–75. ©2010 AACR.*

Introduction

Deregulation of the cell cycle is one of the hallmarks of cancer and is governed by the core cell cycle proteins such as retinoblastoma (pRb) and cyclin-dependent kinases (CDK; ref. 1). Although the role of CDKs in cell cycle progression is well established, defining the complete substrate repertoire of CDKs remains an enigma; many of their potential substrates are yet to be identified. In spite of the known redundancy among CDKs, CDK4 and CDK2 cooperatively play an important role in the G_1-S transition as attested by the mid-gestational embryonic lethality of the $CDK2^{-/-}/CDK4^{-/-}$ double-knockout mice and a delayed G_1-S transition (2). Recent studies also found that CDK2 and CDK4 are essential for various oncogene-mediated tumorigenesis and that use of CDK2/CDK4 inhibitors may be a viable option for treatment of tumors with wild-type (WT) p53 (3). Collectively, these emerging studies suggest that phosphorylation of downstream effector proteins by these interphase CDKs plays a crucial event in tumorigenesis.

Estradiol (E2) via the estrogen receptor (ER) promotes cell proliferation in a wide variety of tissues, including mammary glands, and is implicated in breast cancer initiation and progression (4). Estrogen recruits noncycling cells into the cell cycle and promotes G_1 to S cell cycle phase progression. Induction of the early response genes (such as c-*myc* and c-*fos*) is proposed as one mechanism of this process (5–7), whereas regulation of CDK2 and CDK4 activities is proposed as another (8–10). In addition, cyclin D1 was identified as a target of E2 action, and estrogen treatment was shown to upregulate cyclin D1 levels (11). However, the molecular mechanisms underlying E2 regulation of G_1-S phase transition are not completely understood.

PELP1 (proline-, glutamic acid–, and leucine-rich protein 1), a nuclear receptor coregulator, plays an important role in ER signaling (12). PELP1 is a recently discovered proto-oncogene (13) that exhibits aberrant expression in many hormone-related cancers (12) and is a prognostic indicator of shorter breast cancer–specific survival and disease-free intervals when overexpressed (14). PELP1 seems to function as a scaffolding protein with no known enzymatic activity (12), and the mechanism by which PELP1 promotes oncogenesis remains elusive. We have previously shown that PELP1 overexpression promotes E2-mediated G_1-S progression (15).

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Although these findings suggest that PELP1 may play a role in cell cycle progression, little is known about the molecular mechanism(s) responsible for its oncogenic function.

In this study, we identified PELP1 as a novel substrate of CDKs and found that CDK phosphorylation is important for the proper function of PELP1 in modulating hormone-driven cell cycle progression and also for optimal E2F transactivation function. Our findings revealed a novel mechanism by which CDKs use nuclear receptor coregulators to assist cell cycle progression.

Materials and Methods

Cell lines and reagents

Human breast cancer cells MCF7, ZR75, IMR-90, NIH3T3, and 293T were obtained from the American Type Culture Collection. All stable cell lines were generated through 500 μg/mL G418 (neomycin) selection. E2 was purchased from Sigma. The PELP1 antibody was from Bethyl Laboratories. Recombinant enzyme complexes (CDK4/cyclin D1, CDK2/cyclin E, and CDK2/cyclin A) and CDK antibodies were purchased from Cell Signaling Technology. Anti–green fluorescent protein (GFP) antibody was purchased from Clontech. PELP1 SMARTpool small interfering RNA (siRNA) was purchased from Dharmacon. Phospho-PELP1 antibody was generated by Open Biosystems (Thermo Fisher Scientific) against phospho-PELP1 Ser^{991} [peptide sequence TLPPALPPPE(pS)PPKVQPEPEP]. PELP1 220B2 antibody was generated by University of Texas Health Science Center core facility. The plasmids glutathione *S*-transferase (GST)–PELP1 deletions (16), E2F-Luc (17), and GFP-PELP1 (16) were described previously. Expression vectors for *p16INK4A, CDK4, CDK2*, and *cyclin E* were purchased from Addgene, Inc. Expression vectors for E2F1 and DP1 were purchased from Origene. The PELP1 CDK site mutations were generated on either pGEX-GST-PELP1 deletions or GFP-PELP1 backbone by site-directed mutagenesis (QuikChange Mutagenesis kit, Stratagene). Yeast two-hybrid screening was performed as described (18).

CDK phosphorylation assays

All *in vitro* kinase assays using CDK4 and CDK2 enzymes were performed using the kinase buffer comprising 60 mmol/L HEPES-NaOH (pH 7.5), 3 mmol/L $MgCl_2$, 3 mmol/L $MnCl_2$, 3 μmol/L sodium orthovanadate, 1.2 mmol/L DTT, 10 μCi [γ-^{32}P]ATP, and 100 μmol/L cold ATP and the purified enzyme complex (100–200 ng/30 μL reaction). Bacterially or insect cell purified GST-tagged full-length PELP1 and deletions were used as substrates for the *in vitro* CDK kinase assays. Each reaction was carried out for 30 minutes at 30°C and stopped by addition of 10 μL of 4× SDS buffer.

Reporter gene assays

Reporter gene assays were performed by transient transfection using FuGENE6 method (Roche) as described (17). Briefly, cells were transfected using 500 ng of E2F-Luc reporter, 50 ng *E2F*, 50 ng *DP1*, and 10 ng pSV β-galactosidase, with or without 200 ng of PELP1-WT or PELP1-CDK site mutant (MT) expression vectors. Cells were lysed in passive lysis buffer 36 to 48 hours after transfection, and the luciferase assay was performed using a luciferase assay kit (Promega). Each transfection was carried out in six-well plates in triplicate and normalized with either β-galactosidase activity or the total protein concentration.

Real-time PCR and cell cycle microarray

Cells were harvested with Trizol Reagent (Invitrogen), and total RNA was isolated according to the manufacturer's instructions. cDNA synthesis was done using SuperScript III RT-PCR kit (Invitrogen). Real-time PCR was done using a Cepheid SmartCycler II with specific real-time PCR primers for the E2F target gene (Supplementary Table S1). Results were normalized to actin transcript levels, and the difference in fold expression was calculated using the $\Delta\Delta C_T$ method. Cell cycle microarray was purchased from SABiosciences, and analysis was performed as per the manufacturer's instructions.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) analysis was performed as described previously (19). MCF7 or ZR75 cells expressing GFP-PELP1 WT or MT were cross-linked using formaldehyde, and the chromatin was subjected to immunoprecipitation using the indicated antibodies. Isotype-specific IgG was used as a control. DNA was resuspended in 50 μL of Tris-EDTA buffer and used for PCR amplification using the specific primers (Supplementary Table S1).

Cell cycle analysis, cell synchronization, and cell proliferation assays

IMR-90 and NIH3T3 cells were synchronized to G_0-G_1 phase by serum deprivation for 3 days and released into the cell cycle by addition of 10% fetal bovine serum–containing medium. MCF7, ZR75, and other derived model cell lines were synchronized to G_0-G_1 phase by serum starvation for 3 days in 0.5% dextran-coated charcoal-treated serum-containing medium and released into the cell cycle by addition of 10^{-8} mol/L E2. Double-thymidine block was done to arrest model cells at late G_1 phase (20). Flow cytometry was performed to analyze the cell cycle progression as described previously (15). Cell proliferation rate was measured by using a 96-well format with CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions.

Immunofluorescence, confocal microscopy, and immunohistochemical studies

Cellular localization of PELP1-WT or PELP1-MT was determined by indirect immunofluorescence as described previously (19). Immunohistochemistry was performed using a method as described (21).

Tumorigenesis assays

For tumorigenesis studies, model cells (5×10^6) were implanted s.c. into the flanks of 6- to 7-week-old female nude mice (n = 6 per group) as described (22). Each mice received one 60-day release E2 pellet containing 0.72 mg E2

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(Innovative Research of America) 2 days before implantation of cells, and tumors were allowed to grow for 6 weeks. Tumor volumes were measured with a caliper at weekly intervals. After 6 weeks, the mice were euthanized, and the tumors were removed, weighed, and processed for immunohistochemical staining. Tumor volume was calculated using a modified ellipsoidal formula: tumor volume = 1/2 ($L \times W^2$), where L is the longitudinal diameter and W is the transverse diameter (23, 24).

Results

PELP1 is a novel substrate of interphase CDKs

To examine the significance of PELP1 in cell cycle progression, we first analyzed the expression profile of PELP1 throughout the cell cycle using two normal cell lines, IMR-90 (human diploid fibroblast cell line) and NIH3T3 (murine fibroblast cell line), using an antibody (220B2) generated in our laboratory (Supplementary Fig. S1). Serum-starved fibroblast cells were synchronized to G_1 phase and released into the cell cycle by addition of 10% serum, and the expression of PELP1 was analyzed on a 4% to 12% Bis-Tris gradient gel. In human fibroblast cells, two forms of PELP1 were detected: a slow-migrating form and a fast-migrating form (Fig. 1A, left). In murine fibroblast cells, two slower-moving forms and one faster-moving form were present during various phases of the cell cycle (Fig. 1A, right). The PELP1 slower-migrating bands were also detected by the PELP1 antibody that was previously generated in our lab (15) and was raised against NH_2-terminal epitope amino acids 540 to 560 (Fig. 1A). Intriguingly, these slow-migrating bands were difficult to separate on nongradient gels and escaped detection using existing commercial antibodies (data not shown). Shifted PELP1 bands were also seen in various cancer cell lines when stimulated with 10% serum (Supplementary Fig. S1B). Treatment of the total lysates with λ-phosphatase abolished the slower-migrating forms (Supplementary Fig. S1C). These results suggested that PELP1 may be subjected to posttranslational modification, which may be phosphorylation, during cell cycle progression.

To identify potential kinases that phosphorylate PELP1 during cell cycle progression, we performed a yeast two-hybrid screen using mammary gland cDNA library with PELP1 as bait. One of the proteins identified using this screen was CDK4. We confirmed this interaction with purified plasmids in a yeast-based growth assay. The data indicate that the NH_2 terminus (1–400 amino acids) of PELP1 functions as the CDK4-binding region (Supplementary Fig. S2A). Because PELP1 can participate in E2-mediated cell cycle progression, we used breast cancer model cells to further characterize the PELP1-CDK4 interaction. Using coimmunoprecipitation with PELP1 antibody, we found that PELP1 binds CDK4 *in vivo* (Fig. 1B, left) and E2 stimulation enhances the PELP1 interaction with CDK4 (Fig. 1B, middle). In a GST pull-down assay, GST-CDK4 efficiently interacted with the NH_2 terminus of PELP1, and deletion analysis revealed that 200 to 350 amino acids in PELP1 serve as a docking site for CDK4 (Supplementary Fig. S2B). Using *in vitro* kinase assays with commercially procured CDK4/cyclin D1 and purified GST-tagged PELP1, we found that PELP1 can be efficiently phosphorylated by CDK4 *in vitro* (Fig. 1B, right). Studies have previously shown that E2-mediated cell cycle progression occurs through activation of interphase CDKs (both CDK4 and CDK2; ref. 8). Because the slower-moving forms of PELP1 persisted beyond G_1-S phase (Fig. 1A) and because PELP1 has 11 consensus "Cy" motifs (RXL), which are important for cyclin binding (25, 26), and four CDK2 phosphorylation motifs, we examined whether PELP1 interacts with CDK2 as well. Immunoprecipitation showed that PELP1 interacts with CDK2 (Fig. 1C, left) and that this PELP1-CDK2 interaction occurred at later stages (10–16 hours of E2 stimulation) of cell cycle progression (Fig. 1C, middle). *In vitro* kinase assays using purified CDK2/cyclin E and CDK2/cyclin A complexes further showed that full-length PELP1 may also be a potential substrate of CDK2 (Fig. 1C, right). We further investigated whether CDKs phosphorylate PELP1 *in vivo* by cotransfecting PELP1 with or without CDK4/cyclin D1 and with natural or synthetic inhibitors of CDKs into 293T cells. Cells were metabolically labeled with [^{32}P]orthophosphoric acid, and PELP1 phosphorylation was measured by using autoradiography after immunoprecipitation. Cotransfection of CDK4/cyclin D1 with PELP1 stimulated phosphorylation of PELP1, whereas cotransfection of the natural CDK4 inhibitor p16INK4 decreased CDK4/cyclin D1–mediated PELP1 phosphorylation (Supplementary Fig. S3A). Both the CDK4 inhibitor Ryuvidine (27) and the CDK2 inhibitor Roscovitine (28) substantially reduced PELP1 phosphorylation (Supplementary Fig. S3B and C), suggesting that PELP1 is a novel substrate of interphase CDKs.

CDKs phosphorylate PELP1 at distinct sites

We next mapped the CDK phosphorylation sites in PELP1 using a deletion and mutagenesis approach. PELP1 was expressed and purified as four small GST fusion domains, which were used as substrates for the *in vitro* kinase assay. CDK4/cyclin D1 phosphorylated PELP1 domains containing 400 to 600 amino acids (Fig. 2A, left), whereas CDK2/cyclin E had a preference toward the PELP1 960– to 1130–amino acid fragment (Fig. 2A, middle). Interestingly, CDK2/cyclin A2 uniquely phosphorylated PELP1 400 to 600 amino acids but had no activity in the PELP1 960 to 1130 fragment (Fig. 2A, right). We then mutated all the putative consensus CDK sites (S/T.P) and identified specific sites phosphorylated by each of three CDK complexes. We found that CDK4/cyclin D1 preferentially phosphorylated Ser^{477}, CDK2/cyclin E phosphorylated Ser^{991}, and CDK2/cyclin A2 phosphorylated Ser^{477} (Fig. 2B). We also confirmed that these mutations in the context of full-length PELP1 significantly reduce CDK2/cyclin E– and CDK4/cyclin D1–mediated PELP1 phosphorylation when measured by using an *in vivo* orthophosphate labeling assay (Supplementary Fig. S3D).

To further characterize the *in vivo* relevance of the identified sites, we made an attempt to generate rabbit polyclonal phospho-specific antibodies against each of phospho-Ser^{991} and phospho-Ser^{477} sites. We were successful only in obtaining

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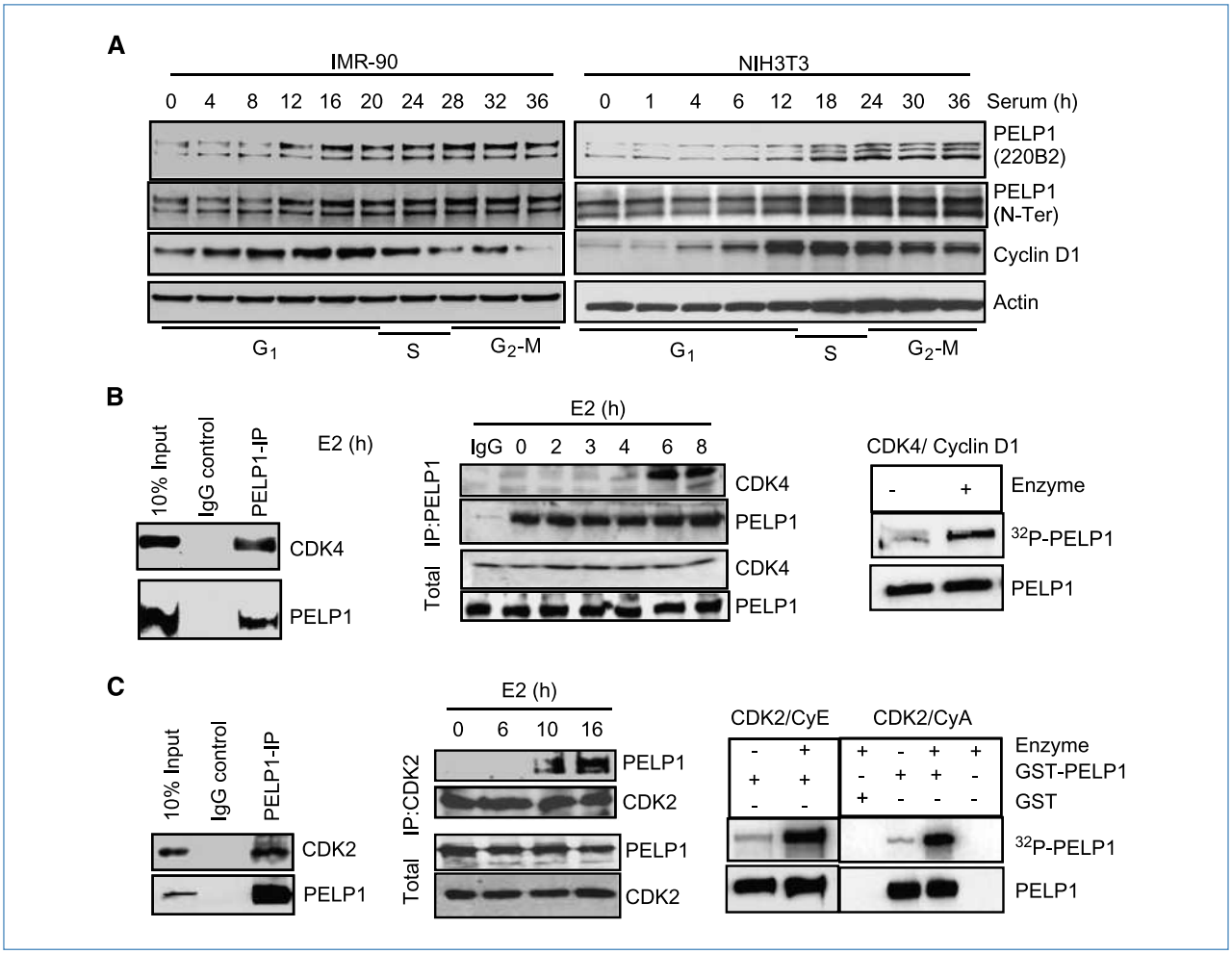

Figure 1. PELP1 is a novel substrate of interphase CDKs. A, expression status of PELP1 in the cell cycle was analyzed in IMR-90 cells (left) and NIH3T3 cells (right). Cells synchronized at G_0-G_1 phase were released into the cell cycle, and lysates from different time intervals were used in Western blot analysis with the 220B2 PELP1 antibody. B, total lysates from MCF7 cells grown in 10% serum were subjected to immunoprecipitation (IP) using the PELP1 antibody (left), and the CDK4 interaction was verified by Western blotting. Middle, T7-tagged PELP1-overexpressing MCF7 cells were treated with 10^{-8} mol/L estrogen for various periods of time, PELP1 was immunoprecipitated, and the CDK4 interaction was verified by Western blotting. Right, *in vitro* kinase assays for the CDK4/cyclin D complex using baculovirus-expressed, GST-tagged, full-length PELP1 as a substrate, and phosphorylation was measured by the amount of ^{32}P incorporation. C, left, total lysates from MCF7 cells grown in 10% serum were subjected to immunoprecipitation using the PELP1 antibody and the CDK2 interaction was verified by Western blot analysis. Middle, MCF7 cells were treated with E2 for various periods of time, and the PELP1 interaction with CDK2 was analyzed by using immunoprecipitation. Right, *in vitro* kinase assays using CDK2/cyclin E (CyE) complex and CDK2/cyclin A2 (CyA) complex using full-length PELP1 as a substrate.

phospho-Ser^{991} antibody, whereas we failed to generate phospho-Ser^{477} antibody because of the poor antigenicity of the Ser^{477} peptide. The antibody was specific to PELP1 Ser^{991} phosphorylation, as its recognition was efficiently competed by phosphorylated peptide but not by unphosphorylated peptide (Fig. 2C, left). Further, it efficiently recognized phosphorylated endogenous and GFP-tagged PELP1 but was unable to detect the Ser^{991} to Ala PELP1-MT (Fig. 2C, left). λ-Phosphatase–treated ZR75 lysates also abolished the ability of the phospho-Ser^{991} antibody to recognize phosphorylated PELP1 in these cells (Supplementary Fig. S4A). E2 stimulation substantially increased the level of serine phosphorylation that is recognized by the Ser^{991} antibody (Fig. 2D, left), and downregulation of ER signaling by antiestrogen ICI 182780 substantially decreased Ser^{991} phosphorylation of PELP1 (Supplementary Fig. S4B). Using double-thymidine block and release of cells, we found that PELP1 Ser^{991} phosphorylation accumulated at the G_1-S boundary and gradually decreased thereafter as the cells progressed into other phases of the cell cycle (Fig. 2D, right). PELP1 Ser^{991} antibody was able to recognize the shifted bands seen in normal IMR-90 cells in gradient gels when synchronized to G_1 phase and released them into the cell cycle (Supplementary Fig. S4D). Overall, these results show that interphase CDKs can phosphorylate PELP1 at Ser^{991} *in vivo*.

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Mutations in CDK2 phosphorylation sites affect PELP1 functions

Because previous studies showed that PELP1 augments G_1-S cell cycle phase progression, we investigated the significance of CDK phosphorylation of PELP1 in cell cycle progression. We constructed an NH_2-terminal GFP-tagged PELP1-MT that lacked these two CDK sites. ZR75 cells stably expressing PELP1-WT and PELP1-MT (pooled clones) were generated. Compared with other ER^+ breast cancer cells such as MCF7, ZR75 cells express low endogenous PELP1 and therefore represent good model to study the effect of mutations by overexpression. In general, these stable clones express 3- to 4-fold more PELP1-MT than endogenous PELP1, and MT expression is equivalent to GFP-tagged PELP1-WT clone (Fig. 3A). Both PELP1-WT and PELP1-MT are localized in the nuclear compartment in cells and migrated to the

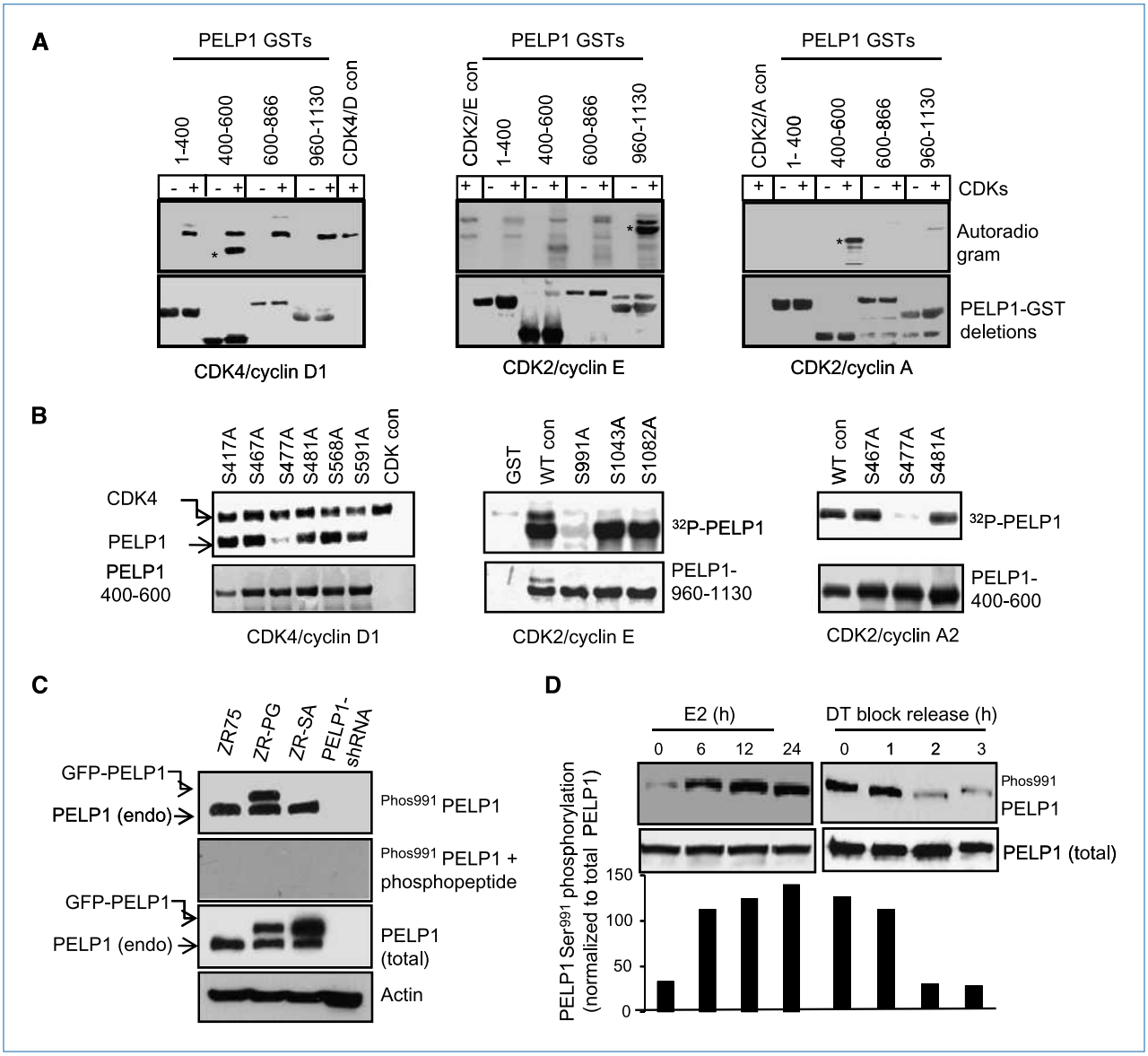

Figure 2. Identification of phosphorylation sites and generation of phospho-specific antibody. A, bacterially expressed GST-PELP1 deletions were used as substrates for an *in vitro* kinase assay using CDK4/cyclin D1 (left), CDK2/cyclin E (middle), and CDK2/cyclin A2 (right), and PELP1 domains phosphorylated by each kinase complex were identified by using autoradiography. B, identification of CDK phosphorylation sites using site-directed mutagenesis (serine to alanine). Various single MTs in the region of interest were used along with respective PELP1-WT deletion fragments. Loss of ^{32}P incorporation revealed the successful identification of phosphorylation sites. C, Western blot analysis of native, WT-tagged PELP1 and tagged phospho-PELP1-MT with the Ser^{991} phospho-PELP1 antibody in the presence or absence of the phosphopeptide. D, MCF7 cells were either arrested and released by E2 stimulation (left) or synchronized into the G_1-S boundary by double-thymidine block and released into the cell cycle by addition of thymidine-free medium (right), and phosphorylation status of PELP1 was analyzed by using the phospho-Ser^{991} antibody.

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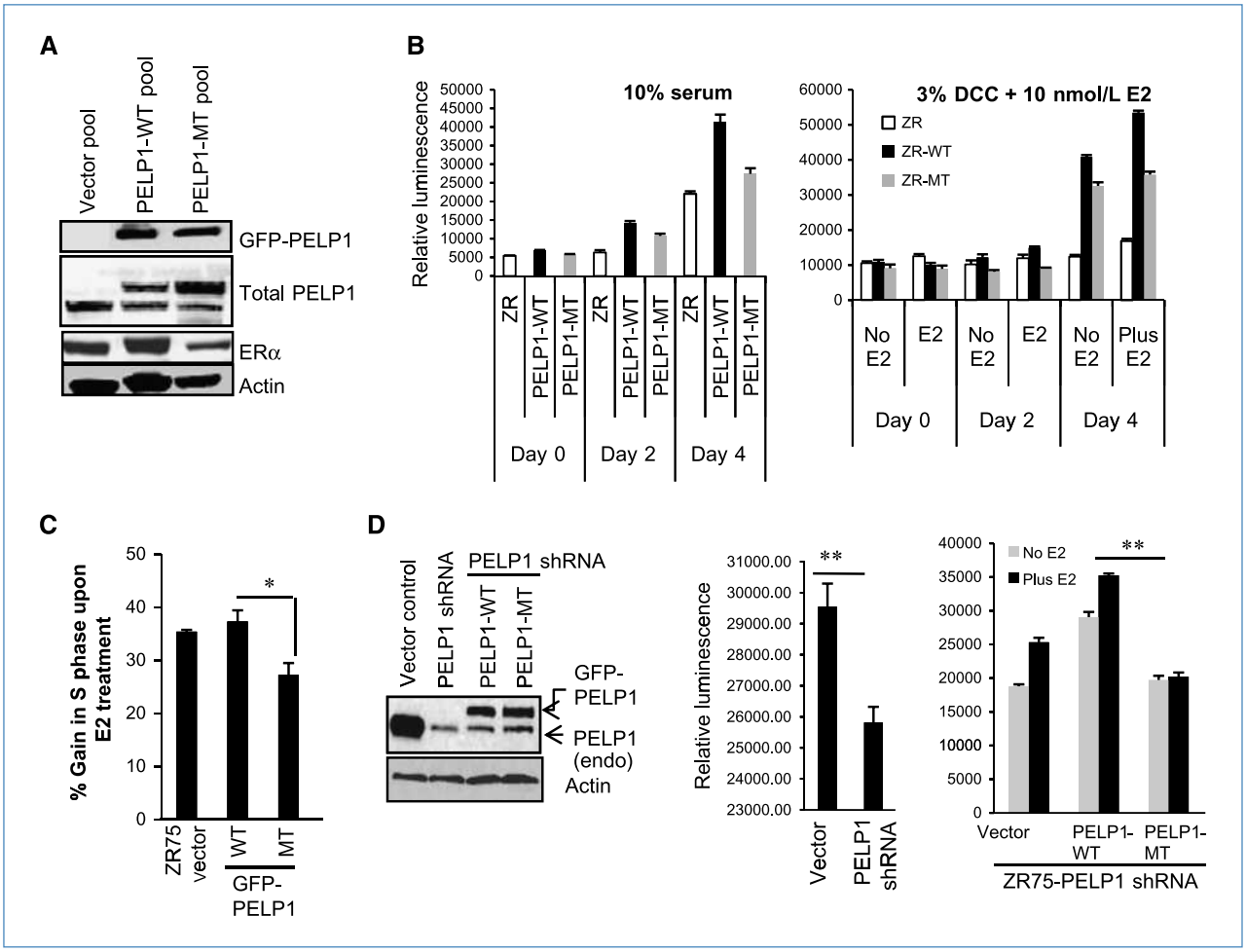

Figure 3. CDK-mediated phosphorylation is required for optimal PELP1 function in cell cycle. A, Western blot analysis of ZR75 cells stably expressing GFP vector, GFP-PELP1-WT, and GFP-PELP1-MT. B, cell proliferation capacity of parental ZR75, PELP1-WT, and PELP1-MT stable cells was analyzed after treating the cells with 10% serum (left) and with or without E2 (right) using CellTiter-Glo assay. C, flow cytometric analysis of cell cycle phases in ZR75-GFP, PELP1-WT, and PELP1-MT pool clones was done and changes observed in S-phase cells are depicted by percentages. D, ZR75 cells that stably express PELP1 shRNA were transfected with shRNA-resistant PELP1-WT or PELP1-MT expression vectors by using nucleofector protocol. After 48 h, transfected cells were subjected to cell proliferation with or without E2 and assayed for proliferation using CellTiter-Glo assay. *, $P < 0.05$; **, $P < 0.001$.

expected size on SDS-PAGE when detected using the GFP antibody (Fig. 3A, left; Supplementary Fig. S5). Western blot analysis using PELP1 phospho-specific antibody showed that GFP-tagged PELP1-WT was phosphorylated during cell cycle progression, whereas PELP1-MT had no signs of phosphorylation (Supplementary Fig. S4C, right). As expected from previous studies, PELP1-WT expression increased cell proliferation as measured by using a CellTiter-Glo assay and bromodeoxyuridine incorporation, whereas mutations in PELP1 diminished its ability to increase cell proliferation (Fig. 3B; Supplementary Fig. S5B). We then analyzed the cell cycle progression of the PELP1-WT and PELP1-MT clones by flow cytometry and found that PELP1-WT expression contributed to increased G_1-S progression, whereas mutation of the CDK sites in PELP1 diminished the number of cells entering S phase on E2 stimulation (Fig. 3C). We then used a knockdown/replacement strategy to validate the effect of CDK phosphorylation on PELP1-mediated proliferation. ZR75 cells that stably express PELP1 short hairpin RNA (shRNA) were transfected with shRNA-resistant PELP1-WT or PELP1-MT expression vectors using nucleofector protocol (Amaxa) that achieved >90% transfection efficiency. After 48 hours, transfected cells were allowed to proliferate with or without E2. Results showed that PELP1-WT, but not PELP1-MT, enhanced E2-mediated proliferation (Fig. 3D). These findings suggest that phosphorylation by CDKs is biologically relevant for PELP1-mediated G_1-S phase transition.

CDK-PELP1 axis modulates expression of E2F target genes

Because PELP1 is a pRb-binding protein and CDKs phosphorylate PELP1, we investigated whether CDK phosphorylation of PELP1 aids E2F functions. First, we compared the expression of genes involved in cell cycle progression

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between MCF7 and MCF7-PELP1 shRNA-stable cells using the cell cycle microarray that contains 112 genes involved in cell cycle regulation. Target genes whose expression was differentially regulated (with at least a 2-fold difference) on PELP1 depletion were identified. Downregulation of PELP1 substantially reduced the expression of several cell cycle genes, including *cyclin D1, pRb, cyclin B2*, and *CDC25C* (Fig. 4A). We then examined whether PELP1 enhances E2F-mediated gene activation and whether CDK phosphorylation affects PELP1 activation of E2F functions by using an E2F-Luc reporter. PELP1 knockdown substantially reduced the E2F reporter gene activity (Fig. 4B, left). The cells with PELP1-WT overexpression had greater E2F-Luc reporter activity compared with vector-transfected cells, whereas PELP1-MT that lacked CDK phosphorylation sites failed to enhance the E2F reporter activity (Fig. 4B, right). ChIP showed that phosphorylated PELP1 is recruited to the promoters of the E2F target gene promoters cyclin A and cyclin E, both of which contain E2F-binding sites (Fig. 4C). However, mutation of CDK phosphorylation sites in PELP1 did not affect its recruitment over E2F target genes (Supplementary Fig. S5C). However, real-time quantitative PCR analysis showed that PELP1 knockdown significantly decreased the expression of several E2F target genes in both MCF7 and ZR75 cells (Fig. 4D). These results suggest that the phosphorylation of PELP1 by CDKs plays a critical role in the expression of E2F target genes.

Mutation of CDK sites decreases PELP1 oncogenic potential *in vivo*

We used a nude mouse xenograft model to examine whether CDK phosphorylation of PELP1 is required for tumorigenic potential of breast cancer cells *in vivo* using model cells that express either PELP1 or PELP1-MT that lack CDK phosphorylation sites. Both vector-transfected and PELP1-WT–expressing cells formed tumors, and these tumors grew linearly with time (Fig. 5A). However, PELP1-MT–injected sites had smaller tumors than those that developed in the controls (Fig. 5B). Compared with both MT and parental ZR75 cells, ZR75-PELP1-WT cells had more PELP1 phosphorylation at Ser^{991} (Fig. 5C). Proliferating cell nuclear antigen (PCNA) staining of the tumor sections revealed greater

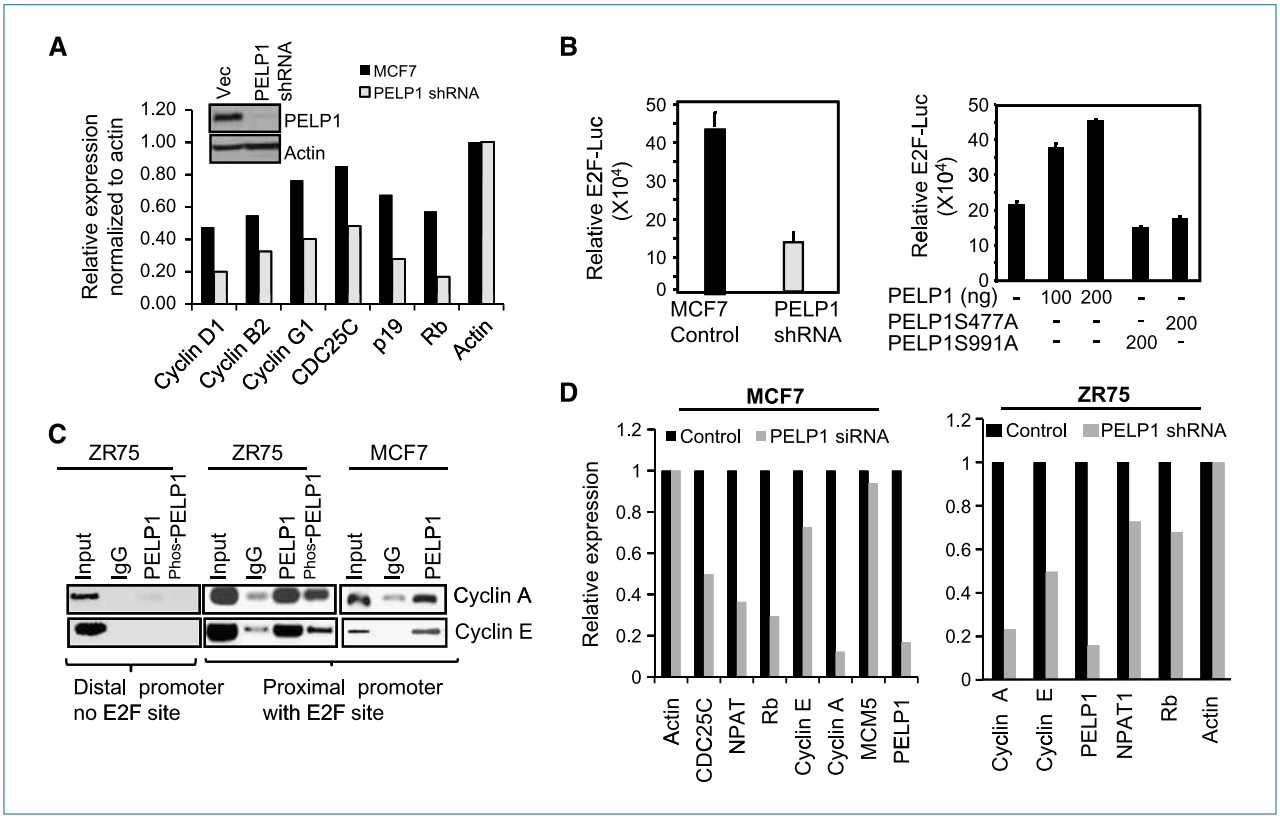

Figure 4. CDK-dependent PELP1 phosphorylation modulates E2F transactivation functions. A, RNA isolated from MCF7 control and stable MCF7-PELP1 shRNA-expressing cells was hybridized to the human cell cycle microarray. Changes in the gene expression were analyzed using SABiosciences software with actin as a control for normalization. Representative genes downregulated on PELP1 depletion are shown. Inset, expression of PELP1 in PELP1 shRNA clones. B, left, MCF7 and MCF7-PELP1 shRNA cells were transfected with E2F-Luc reporter along with E2F and DP1 plasmids, and luciferase activity was measured after 36 h. NIH3T3 cells were transfected with E2F-Luc with E2F and DP1 plasmids and with or without PELP1-WT or PELP1-MT. Right, luciferase activity was measured after 36 h of transfection. C, recruitment of PELP1 to E2F target gene promoter was analyzed by using the ChIP assay with MCF7 and ZR75 cells. D, left, MCF7 cells were transiently transfected with control or PELP1-specific siRNA; right, ZR75 cells were stably transfected with control or PELP1-specific shRNA. Total RNA was isolated and expression of classic E2F target genes was analyzed by real-time quantitative PCR.

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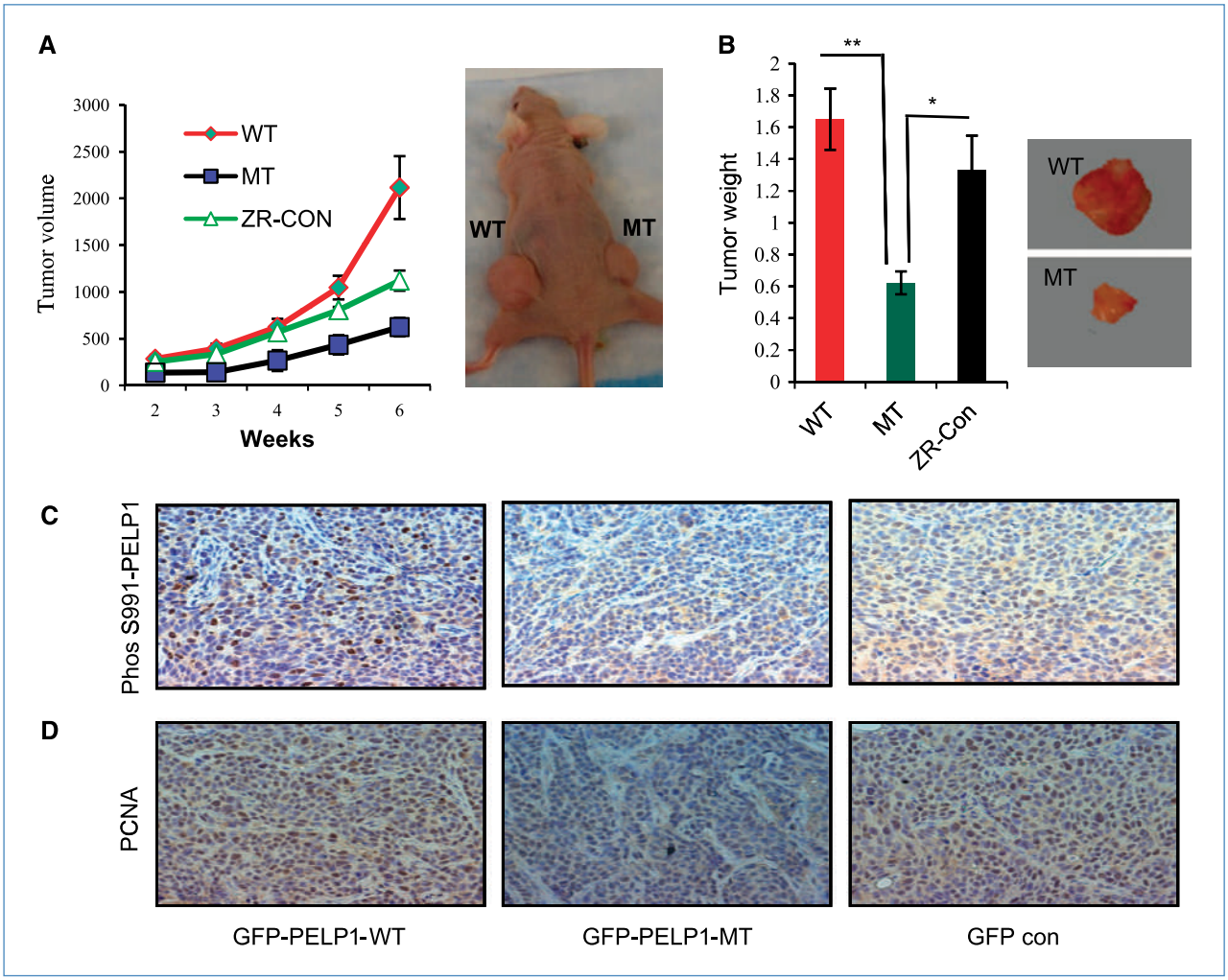

Figure 5. CDK phosphorylation is essential for PELP1 oncogenic function. A, nude mice implanted with E2 pellet were injected s.c. with ZR75-GFP, ZR75-PELP1-WT, or ZR75-PELP1-MT cells and tumor growth was measured at weekly intervals. Left, tumor volume. B, average tumor weight. Representative images of tumors are shown. Status of PELP1 phosphorylation (C) and PCNA expression as a marker of proliferation (D) was analyzed by immunohistochemistry. *, $P < 0.05$; **, $P < 0.001$.

proliferation in the PELP1-WT xenograft tumors than in the PELP1-MT tumors (Fig. 5D). These results suggested that CDK phosphorylation of PELP1 is essential for optimal growth of E2-driven tumor growth *in vivo*.

Discussion

E2 is known to promote key cell cycle events such as activation of CDK and hyperphosphorylation of pRb in ER-positive breast epithelial cells, leading to increased rate of G_1-S phase transition (8). Both CDK4 and CDK2 drive G_1-S transition in the cell cycle, and their expression is deregulated in tumors, indicating that phosphorylation of downstream effector proteins by CDKs is vital in tumorigenesis (3). We found that (*a*) PELP1 phosphorylation changes during cell cycle progression, (*b*) PELP1 interacts with the G_1-S phase CDKs (both CDK4 and CDK2), (*c*) PELP1 couples E2 signaling to the E2F axis, (*d*) PELP1 is a novel substrate of CDKs, and (*e*) CDK phosphorylation plays a key role in PELP1 oncogenic functions. Collectively, these results suggest that phosphorylation of PELP1 by CDKs confers a growth advantage to breast epithelial cells and thus contributes to tumorigenesis by accelerating cell cycle progression.

Our results identified ER coregulator PELP1 as another novel substrate of CDKs and that its phosphorylation is essential for optimal cell cycle progression. CDKs phosphorylate PELP1 minimally at two distinct sites (Ser^{477} and Ser^{991}), and these sites are phosphorylated by distinct CDK/cyclin complexes. We only validated Ser^{477} and Ser^{991} as *in vivo* sites for CDK4 and CDK2, respectively. It is possible that there may be additional putative minor sites that could be phosphorylated *in vivo*, and we will explore those possibilities in future studies. Ser^{477} was previously identified as a site of phosphorylation of PELP1 in a large screen for epidermal growth factor

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(EGF)–stimulated phosphoproteins (29), and interestingly, EGF is shown to activate CDK4/cyclin D1 complexes (30). We speculate that Ser^{477} could be the site that facilitates ER/CDK4 cross talk, which occurs during mammary gland development and also in pathologic settings including breast cancer progression. Reinforcement of Ser^{477} phosphorylation by CDK4/cyclin D1 and CDK2/cyclin A2, two kinases in two different cell cycle phases, is quite intriguing and may be important for unknown reasons.

Evolving evidence suggests that PELP1 may function as a large scaffolding protein, modulating gene transcription with protein-protein interactions. The NH_2 terminus of PELP1 interacts with ERα, pRb, and Src (12). Our results suggest that the NH_2 terminus of PELP1 also harbors a binding site for CDK4 that facilitates PELP1 phosphorylation. We found that during cell cycle progression, PELP1 runs as two to three slower-migrating bands and has a molecular weight of >160 kDa. Posttranslational modification of PELP1 probably accounts for the multiple shifts of PELP1 observed on denaturing gradient gels during cell cycle progression, as previously described for pRb (31). We also showed that PELP1-WT, but not PELP1-MT, overexpression promoted progression of breast cancer cells to S phase and that PELP1-MT significantly reduced E2-mediated *in vivo* tumorigenic potential. Our findings suggest that CDK phosphorylation of PELP1 plays a permissive role in E2-mediated cell cycle progression, presumably via its regulatory interaction with the E2F pathway.

Coregulators are often recruited by transcription factors to mediate epigenetic modifications at target gene promoters; for example, E2F uses coregulators such as HCF1 (32) and KAP1 (33) to facilitate gene activation and repression, respectively. PELP1 can interact not only with histone-modifying acetylases and deacetylases (34) but also with histone-modifying methyltransferases and demethylases. A recent study identified PELP1 as a component of the MLL1 methyltransferase complex (35), and we have found that PELP1 functions as a reader of dimethyl-modified histones (36). Our results from the cell proliferation assays using PELP1-MT cells established the significance and role of CDK phosphorylation of PELP1 in cell cycle progression. Collectively, these results suggest that PELP1 serves as a key coregulator that connects E2 signaling to the activation of E2F target genes probably by facilitating epigenetic changes, which will be addressed in future studies.

PELP1 expression is deregulated in metastatic tumors (13). PELP1 protein expression is an independent prognostic predictor of shorter breast cancer–specific survival, and its elevated expression is positively associated with markers of poor outcome (14). Our data suggest that ER-CDK-PELP1 signaling plays a role in E2-mediated cell cycle progression and that the CDK deregulation commonly seen in breast tumors may play a role in metastasis by enhancing E2-mediated cell cycle progression via excessive phosphorylation of PELP1. Deregulation of both CDKs and PELP1 in breast cancer suggests that the modulation of PELP1 pathway by CDKs may represent a potential mechanism by which CDKs promote breast tumorigenesis.

In summary, our data provide the first evidence showing PELP1 as a novel substrate of CDKs. We also provide evidence to indicate that PELP1 phosphorylation by CDKs is essential for optimal E2-mediated cell cycle progression. On the basis of these findings, we predict that phosphorylation of PELP1 by CDKs confers a growth advantage to breast epithelial cells by activating the pRb/E2F pathway and thus contributes toward tumorigenesis by accelerating cell cycle progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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