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Cyclin D1 determines estrogen signaling in the mammary gland in vivo.

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Cyclin D1 Determines Estrogen Signaling in the Mammary Gland In Vivo

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The *CCND1* gene, which is frequently overexpressed in cancers, encodes the regulatory subunit of a holoenzyme that phosphorylates the retinoblastoma protein. Although it is known that cyclin D1 regulates estrogen receptor (ER)α transactivation using heterologous reporter systems, the in vivo biological significance of cyclin D1 to estrogen-dependent signaling, and the molecular mechanisms by which cyclin D1 is involved, are yet to be elucidated. Herein, genome-wide expression profiling conducted of 17β-estradiol-treated castrated virgin mice deleted of the *Ccnd1* gene demonstrated that cyclin D1 determines estrogen-dependent gene expression for 88% of estrogen-responsive genes in vivo. In addition, expression profiling of 17β-estradiol-stimulated cyclin D1 small interfering RNA treated MCF7 cells shows cyclin D1 is required for estrogen-mediated gene expression in vitro. Genome-wide chromatin immunoprecipitation-Seq analysis revealed a cyclin D1-DNA bound form associated with genes that were regulated by estrogen in a cyclin D1-dependent manner. The cyclin D1-dependent estrogen signaling pathways identified in vivo were highly enriched for extracellular membrane-associated growth factor receptors (epidermal growth factor receptor, ErbB3, and EphB3) and their ligands (amphiregulin, encoded by *AREG* gene), and matrix metalloproteinase. The AREG protein, a pivotal ligand for epidermal growth factor receptors to promote cellular proliferation, was induced by cyclin D1 via the *AREG* promoter. Chromatin immunoprecipitation analysis demonstrated the recruitment of cyclin D1 to the breast cancer 1 (Brca1)/ERα binding site of the *Areg* gene. *Cyclin D1* genetic deletion demonstrated the in vivo requirement for cyclin D1 in assembling the estrogen-dependent amplified in breast cancer 1-associated multiprotein complex. The current studies define a requirement for cyclin D1 in estrogen-dependent signaling modules governing growth factor receptor and ligand expression *in vivo* and reveal a noncanonical function of cyclin D1 at ERα target gene promoters. Cyclin D1 mediates the convergence of ERα and growth factor signaling at a common *cis*-element of growth factor genes. **(*Molecular Endocrinology* 27: 1415–1428, 2013)**

The human *CCND1* gene was initially cloned as a component of a breakpoint rearrangement in parathyroid adenoma (1). The *CCND1* gene is commonly amplified and/or overexpressed in a variety of human malignancies, including human breast cancer (2). Immunoneutralizing antibody and antisense experiments demonstrated the abundance of cyclin D1 is rate limiting in estrogen-induced DNA synthesis and oncogene-induced contact-independent breast tumor growth in mice (3, 4). *Ccnd1* gene knockout mice demonstrated a nonredundant func-

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Abbreviations: AREG, amphiregulin; AIB1, amplified in breast cancer 1; BRCA1, breast cancer 1; ChIP, chromatin immunprecipitation; CMV, cytomegalovirus; E_2, 17β-estradiol; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen response element; GST, glutathione-*S*-transferase; HMC, HMW complex; HMW, high molecular weight; LMC, LMW complex; LMW, low molecular weight; MMP, matrix metalloprotease; PR, progesterone receptor; pRb, retinoblastoma protein; siRNA, small interfering RNA; SRC, steroid receptor coactivator.

tion for cyclin D1 in mammary gland development (5–8), macrophage migration (9), angiogenesis (10), mammary epithelial cell migration, mitochondrial size and metabolism (11, 12), micro RNA expression (13), and UV radiation sensitivity (14).

The cyclin D1 protein conveys canonical functions as the regulatory subunit of a holoenzyme that phosphorylates target proteins including the pRb protein to inhibit DNA synthesis, and the nuclear respiratory factor 1 (NRF1) protein to inhibit mitochondrial biogenesis (11, 15, 16). Noncanonical function of cyclin D1 includes association with transcription factors and coactivators, which occur independently of the cyclin D1-associated kinase activity (17). Association of cyclin D1 with more than 30 transcription factors (ERα, androgen receptor, and peroxisomal proliferator-activated receptor-γ) and tumor suppressors (cyclin D binding myb-like transcription factor 1 [DMPF1] and breast cancer 1 [BRCA1]) has been demonstrated in cultured cells (17). The abundance of endogenous cyclin D1 determines the recruitment of transcription factors in the context of local chromatin to target sequences in ChIP assays (18, 19). Cyclin D1 facilitates the recruitment of DNA chromatin-modifying enzymes including p300/CREB-binding protein, suppressor of variegation 3–9 homolog (SUV39H), and Heterochromatin Protein 1 Homolog Alpha (HP1α) and cyclin D1 is sufficient to alter local histone acetylation and methylation at target *cis*-elements. In recent studies using promoter chromatin immunoprecipitation (ChIP)-on-ChIP analysis of 12K genes, cyclin D1 was shown to be recruited to the promoter region of a number of genes (19).

The clinical observation that cyclin D1 overexpression is frequently associated with human cancer led to mechanistic analysis of cyclin D1 function in estrogen action. Estrogen induces *CCND1* gene expression (20, 21), and cyclin D1 associates with ERα and the steroid receptor coactivator (SRC)1 (22–24). Estrogen binds ERα to coordinate diverse biological functions, with discrete subcellular locations, including the nucleus, cell membrane, and mitochondria. The ERα conducts both nuclear (genomic) and extranuclear (nongenomic) functions with dissociable patterns of gene expression (reviewed in Ref. 25). In the nucleus, ERα recruits coactivator and corepressor complexes in the context of local chromatin. In addition to local promoter upstream regulatory regions, ChIP-Seq studies have shown that a substantial proportion of DNA-bound ERα occupies downstream promoter regulatory regions, intragenic regions, and sites distal to the proximal 1 kb of the transcriptional start site. Nongenomic actions of 17β-estradiol (E_2)/ERα are conducted via cytoplasmic membrane and mitochondrial pools of ERα and kinase activation by the membrane-associated ERα (26, 27).

The current studies were conducted to examine at a high level of resolution the functional significance of cyclin D1 in estrogen signaling. First, genome-wide expression studies conducted on castrated virgin *Ccnd1*$^{-/-}$ mice treated with E_2 identified a molecular signature of cyclin D1-dependent estrogen signaling that includes 88% of E_2-regulated genes. In the mammary gland, hierarchical clustering demonstrated these genes include growth factors, chemokine receptors, and their ligands. Second, we analyzed genome-wide cyclin D1 ChIP-Seq data, extending prior ChIP-on-ChIP analysis. These studies demonstrate that cyclin D1 occupancy is coincident within the genomic region of 16% of E_2-responsive genes. Third, we show that cyclin D1 signaling converges at a common DNA response element of the amphiregulin gene (AREG) promoter that binds ERα and BRCA1. Collectively these studies provide evidence that cyclin D1 participates in estrogen-regulated gene expression in vivo, governing growth factor and cytokine signaling.

Materials and Methods

Cell culture

MCF7 and human embryonic kidney 293T cells were obtained from American Type Culture Collection (Manassas, Virginia) and maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified atmosphere containing 5% CO_2 at 37°C. Cells were seeded onto tissue culture dishes containing phenol red-free DMEM supplemented with 5% charcoal/dextran-treated fetal bovine serum and cultured for 48 hours before all experimental treatments with hormone.

Ovariectomy and E_2 treatment

All aspects of the care and handling of animals used in this study were approved by the Institute Animal Care and Use Committee at Thomas Jefferson University. Genotyping was performed using PCR and primers directed to the murine *Ccnd1* gene as previously described (11). For studies of E_2 treatment of mice, animals were castrated and E_2 pellets implanted for 7 days (28). Briefly, female *cyclin D1*$^{-/-}$ and *cyclin D1*$^{+/+}$ mice were maintained on 12-hour light and 12-hour dark cycles. Mice were ovariectomized at 5 weeks of age via a dorsal incision under xylazine (5 mg/kg)-ketamine (50 mg/kg) anesthesia. The mice were allowed to recuperate for 2 weeks and then were randomly assigned to either replacement pellets (Innovative Research of America) containing E_2 (0.72 mg, 60-day release) or a pellet containing only the placebo, resulting in 4 mice per group. The mice were then humanely euthanized at day 7 following pellet implantation. Bromodeoxyuridene (BrDu) was injected 6 hours before the mammary glands of *cyclin D1*$^{-/-}$ and *cyclin D1*$^{+/+}$ mice were excised and analyzed by whole mount to compare the morphology. Mammary glands were also fixed and

embedded in paraffin. Sections were cut at 5 μm and stained for Ki67 and BrDU. The uterus of mice was also removed and weighed to validate the efficacy of the ovariectomy and estrogen pellet implantation.

Plasmids, transfections, and reporter assays

The expression vectors for p3xFLAG-cyclin D1, Rous sarcoma virus-*Renilla* luciferase reporter, CMV-Luc, and the cyclin D1 promoter luciferase reporter constructs were described previously (29, 30). The ERα expression constructions in p3xFLAG CMV 10 (Sigma-Aldrich) were derived from pHEGO. The AREG promoter reporter constructs were a generous gift from Dr J. Parvin and were previously described (31). The reporter plasmid estrogen response element $(ERE)_2$-TK Luc contains 2 copies of the vitellogenin A2 estrogen-responsive enhancer (11). DNA and small interfering RNA (siRNA) transfection and luciferase assays were done as previously described (11, 32). Cyclin D1 short hairpin RNA knockdown was conducted according to manufacturer's guidelines (Open Biosystems, Pittsburgh, Pennsylvania). Cells were transfected using either calcium phosphate precipitation or Lipofectamine, and the medium was changed after 5 hours. Luciferase activity was determined after 24 hours. A β-galactosidase (β-gal) reporter plasmid was included to control for transfection efficiency. The fold effect was determined by comparison with the effect of the empty expression vector cassette.

RNA isolation, quantitative real-time PCR, and microarray analysis

Total RNA was isolated from mammary gland of transgenic mice, or MCF7 cells treated with cyclin D1 siRNA or control siRNA and vehicle or E_2 (10 nM) for 24 hours, using Trizol (12). RNA quality was determined by gel electrophoresis. Probe synthesis and hybridization were performed as previously described (8). For in vivo studies, the labeled probe was used to hybridize Affymetrix 430 2.0 arrays (Affymetrix, Santa Clara, California). Analysis of the arrays was performed using the R statistics package and the limma library of the Bioconductor software package. Arrays were normalized using robust multiarray analysis, and *P* value of .05 was applied as statistical criteria for differential expressed genes (normalized and raw data available files deposited at Gene Expression Omnibus [GEO]; GSE48884). For in vitro MCF7 studies, labeled probe was used to hybridize Affymetrix Human Gene 1.0 ST arrays (Affymetrix). Microarray data analysis was conducted using GeneSpring software (Agilent Technologies, Palo Alto, California) (normalized and raw data available files deposited at Gene Expression Omnibus [GEO]; GSE 48989). The differentially regulated genes were then grouped using hierarchical clustering with "complete" agglomeration, and each cluster was further analyzed based upon the known function of the genes contained in the cluster. Expression profiles are displayed using TreeView. Classification and clustering for pathway level analysis employed DAVID functional annotation (33, 34). Quantitative real-time PCR was conducted as previously described (35).

ChIP-sequencing and analysis

The cyclin D1 ChIP-Seq has been described previously (36).

Western blot and Northern blot

The antibodies used in Western blot analysis were to cyclin D1 (DCS-6), pRb (C-15), ERα (H-184), cyclin E (HE-12), CDK (C-22), pS2 (C-20) (Santa Cruz Biotechnology, Santa Cruz, California) and AREG (AF262; R&D Systems, Inc, Minneapolis, Minnesota). Proteins were visualized by the chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, New Jersey).

ELISA

For ELISA, cells were seeded at 80% confluence, and the growth medium was changed 24 hours later to serum-free medium after samples were washed with PBS. The conditioned medium was collected 24 hours later, and supernatant was obtained by centrifugation at 2000 rpm for 5 minutes, followed by filtration through a 0.45-μm-pore-size membrane filter. AREG in the conditioned medium was measured using a human AREG ELISA DuoSet kit (R&D Systems, Inc.) in triplicate, as per the manufacturer's recommendations, and normalized by the total protein levels in the cell lysate of each individual sample. Experiments were conducted at least 3 separate times.

ChIP assay

ChIP analysis was done following a protocol provided by Upstate Biotechnology (Charlottesville, Virginia). MCF7 cells were treated with EtOH and E_2 for 1 hour. Chromatin solutions were precipitated overnight at 4°C using 6 μg anti-ERα (H-184, Santa Cruz Biotechnology), 4 μg anti-Cyclin D1 (HD-11, Santa Cruz Biotechnology), 4 μg anti-FLAG (Santa Cruz Biotechnology), and 4 μg anti-BRCA1 (C-20, Santa Cruz Biotechnology) with rotation. For a negative control, rabbit IgG and mouse IgG were immunoprecipitated. ChIP analysis for each immunoprecipitated protein was conducted on the endogenous *AREG* promoter at the BRCA1, ERα, and negative responding sites (35 cycles of PCR). The following primers were used: BRCA1, sense 5′-TGTCAGGTACTAGCTCCG-3′ and antisense 5′-GACACACGCCCCGCCT-3′; ERE, sense 5′-TTCCTGTCTCCGCTTCATTT-3′ and antisense 5′-ACTGGTGGAATACTGGCATT-3′; Negative, sense 5′-AACTGCTGCACAGCAAAAGG-3′ and antisense 5′-GAGCAAGAACTGGCAGATGG-3′.

Gel filtration

Subconfluent MCF7 cells were washed and harvested in PBS and lysed with a homogenizer in a buffer containing 50 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.5), 1 mM EDTA, 10% glycerol, a cocktail of protease inhibitors (Roche, Indianapolis, Indiana), and ligand where appropriate. Samples were centrifuged at 27 000 $\times$ *g* for 15 minutes at 4°C.

After centrifugation the supernatant was filtered over a 0.22-μm pore size MILLEX-GP filter (Millipore Corp, Billerica, Massachusetts) and fractioned on a Superose 6 HR 10/30 gel filtration column (Pharmacia) preequilibrated with 150 mM NaCl, 50 mM sodium phosphate (pH 7.0), 1 mM dithiothreitol, 10 μg/mL benzamidine, and 0.7% n-octyl-β-D-glucopyranoside (supplemented with ligand where appropriate) at a flow rate of 0.2 mL/min and 400-μL fractions were collected. Column calibration was performed under the same conditions with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The void volume of the column was 7.0 mL.

Cycloheximide treatment methods

MCF7 cells were maintained in phenol-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum at 70% confluence for 48 hours. Cells were then treated with 20 μg/mL cycloheximide in the presence or absence of E_2 (10 nM). After treatments, Western blot analyses were performed using the anti-cyclin D1 antibody. GDI serves as protein loading control. The signal intensity of cyclin D1 was determined by Alpha-Imager (Alpha Innotech, Santa Clara, California).

Results

Cyclin D1 governs E_2-dependent epithelial cell growth in mammary gland

In order to characterize the biological significance of cyclin D1 to ERα signaling in the mammary gland in vivo, nonpregnant virgin *Ccnd1*$^{-/-}$ mice were examined and subjected to ovariectomy. After 14 days, when atrophy of the uterus occurred, estrogen pellets were implanted (Figure 1A). After a subsequent week of estrogen replacement, animals were euthanized, and the mammary glands were analyzed. The uteri of *Ccnd1*$^{-/-}$ mice decreased in size upon ovariectomy but increased partially in the presence of E_2 (Figure 1B). The proliferative effect of estrogen was partially dependent upon cyclin D1 as previously described in the uterus (37). The mammary epithelial cells of female mice undergo massive proliferation in response to hormone stimulation during pregnancy. However, the *Ccnd1*$^{-/-}$ mice displayed a severe impairment of mammary gland expansion. The *Ccnd1*$^{+/+}$ mice developed the alveolar lobules, and the density of mammary epithelium was increased upon E_2 treatment (Figure 1C, left panel). In contrast, the *Ccnd1*$^{-/-}$ failed to respond to E_2 treatment in alveolar lobule development (Figure 1C, right panel).

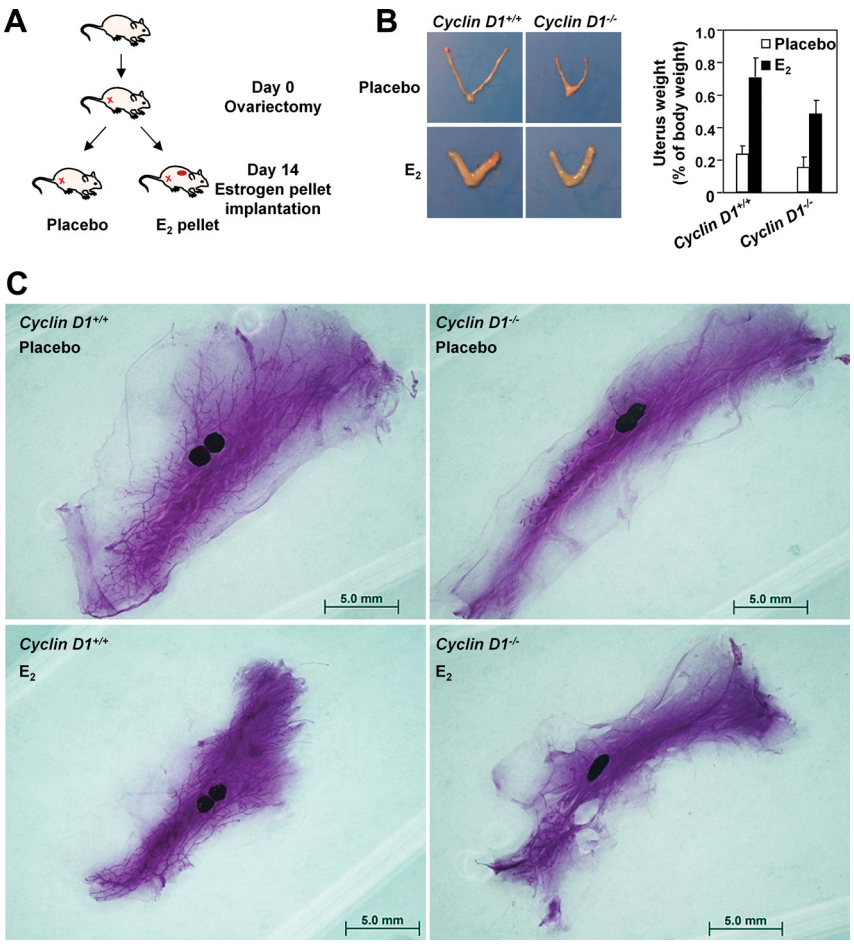

Figure 1. Cyclin D1-Dependent and Independent Function in Estrogen-Regulated Development in Vivo. A, Schematic depicting experimental procedure for ovariectomy and estrogen pellet implantation (n = 16 female mice). Mice were implanted with an estrogen pellet or placebo pellet 14 days after ovariectomy. Tissues were harvested at day 21. B, The representative images of uterus from *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ mice with or without estrogen treatment. Graph depicts uterus weights as a percentage of body weight in *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ mice with or without estrogen treatment. C, Mouse mammary gland whole mounts stained with Carmine dye.

Mammary epithelial cells were isolated from the mammary gland, and the RNA and protein were subjected to analysis. The gene expression profile confirmed the induction of genes previously characterized as estrogen responsive including the oxytocin receptor (Oxtr), epithelial growth factor receptor (Egfr), and growth regulation by estrogen in breast cancer 1 (Greb1) (38). The well-characterized E_2-responsive progesterone receptor (PR) gene was induced 8.4-fold in the wild-type mammary gland and approximately 3-fold in the *Ccnd1*$^{-/-}$. These findings are consistent with the interpretation of a prior study in which the PR was induced by E_2 in the *Ccnd1*$^{-/-}$ mammary gland, but illustrate that endogenous cyclin D1 enhances E_2-mediated induction of PR expression (Supplemental Table 1 published on The Endocrine Journals' Online web site at http://mend.endojournals.org).

Cyclin D1 regulates genes that are E_2-dependent growth factors and matrix metalloproteinases

In the wild-type mammary gland, 608 genes were differentially regulated by E_2 with the vast majority being up-regulated (554 genes) (Fig-

ure 2A and Supplemental Table 1). In contrast, only 134 genes were differentially regulated by E_2 in the *Ccnd1*$^{-/-}$ mice. These results suggest that the vast majority (537 genes; 88%) of E_2-regulated gene expression is dependent upon endogenous cyclin D1 in vivo.

Pathway analysis (PANTHER; Biological Process) was conducted using DAVID bioinformatics resource to determine the gene signaling modules induced by E_2 in a cyclin D1-dependent manner. These analyses demonstrated the estrogen-mediated gene signaling pathways including cellular adhesion, migration, and extracellular matrix (ECM)-linked receptor protein signaling (for ease of comparison, the mean fold induction by E_2 of *Ccnd1*$^{+/+}$ vs *Ccnd1*$^{-/-}$ is shown as a chart to the right of

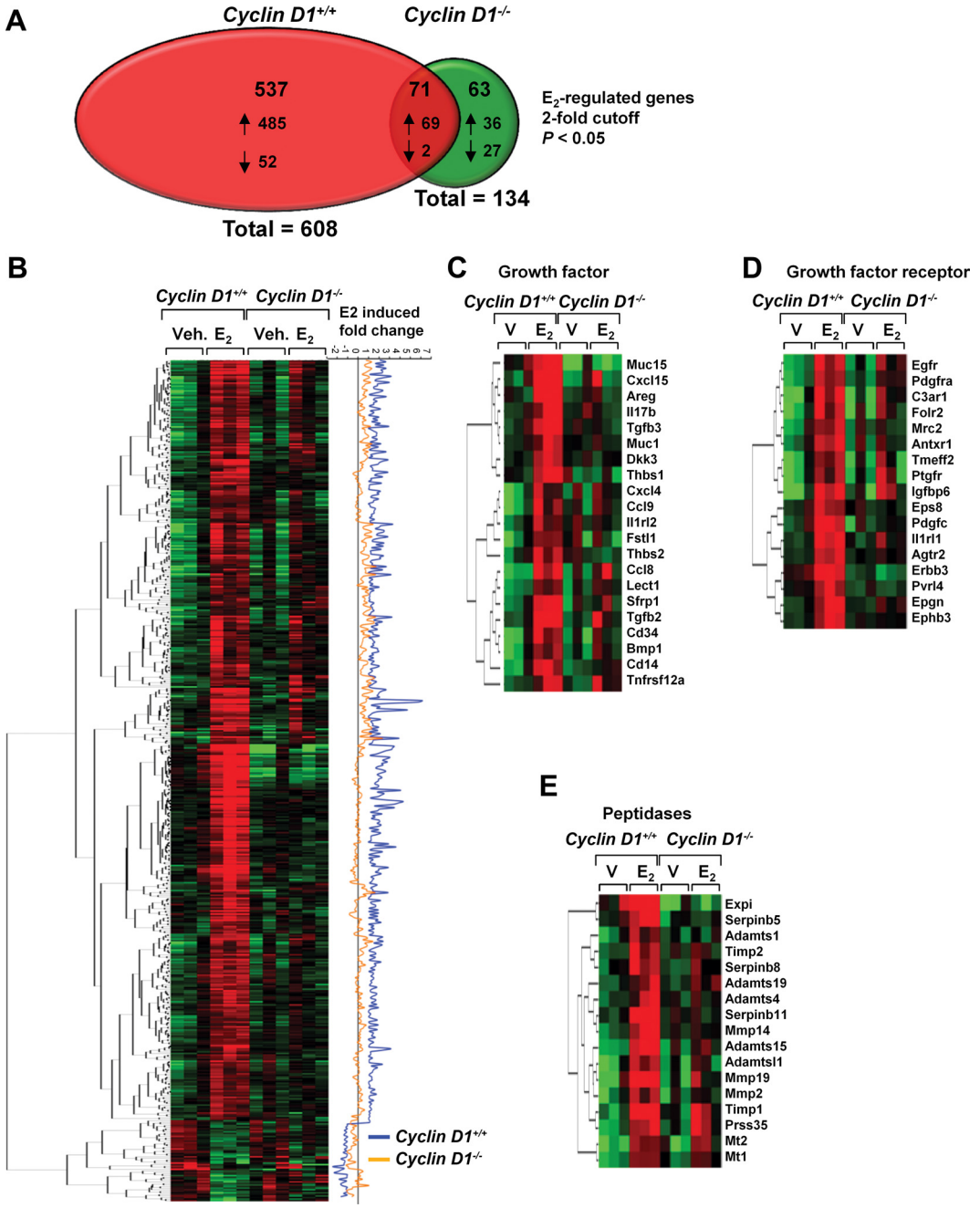

Figure 2. Genome-Wide Profiling of Cyclin D1-Dependent Estrogen-Regulated Genes in Vivo. A, Venn diagram displays the number of genes that were differentially regulated by estrogen in *cyclin D1*$^{+/+}$ mouse mammary glands compared with *cyclin D1*$^{-/-}$ mouse mammary glands. The directionality of change is depicted by up and down arrows. B, Cyclin D1-dependent estrogen-regulated genes were grouped by hierarchical clustering via complete linkage (Cluster 3.0) and visually depicted using Treeview (left). The up-regulated genes are in red and down-regulated genes are in green ($P < .05$). Chart to the right of heat map depicts Log_2 fold change of E_2-induced and -repressed genes comparing *cyclin D1*$^{+/+}$ with *cyclin D1*$^{-/-}$ mouse mammary glands. C–E, DAVID analysis was used to classify the pathways differentially regulated by cyclin D1 in E_2-treated mice. Pathways depicted represent member genes from microarray analysis of estrogen-treated *cyclin D1*$^{+/+}$ mouse mammary glands vs *cyclin D1*$^{-/-}$ mouse mammary glands, growth factors (C), growth factor receptors (D), and peptidases (E). V, vehicle; Veh., vehicle.

the heat map) (Figure 2B and Supplemental Figure 1). Examination of individual genes within these pathways identified enrichment for growth factor and cytokine receptors and their ligands (Figure 2, C–E). The individual genes were tabulated as growth factor receptors (ie, Egfr, Erbb3, and Pdgfr), growth factor ligands (ie, Areg), members of protease-regulatory protein families (a disintegrin and metalloproteinase with thrombospondin motif (Adamts)), tissue inhibitor of metalloprotease and matrix metalloprotease (MMP) families.

Examination of the individual genes induced by E_2 in a cyclin D1-dependent manner identified a group of genes well known to be induced by estrogen, which include the progesterone receptor (*Pgr*) gene, the estrogen-responsive gene (*c-jun*), and a group of several other genes induced by DNA damage repair (*Perp, TP53 apoptosis effector*), TNFα-induced protein 6, dual specificity phosphatase 4, TNF receptors family member 12A, stanniocalcin 2, interferon-induced protein with tetratricopeptide repeats 1, heat shock protein 1A, nucleoredoxin, and immediate early response 3 (Supplemental Figure 2). These studies suggest that endogenous cyclin D1 participates in estrogen-dependent gene expression including estrogen-responsive genes induced by DNA damage-mediated signaling.

Gene ontology (GO) terms used to define genes induced by E_2 via cyclin D1 encompassed genes involved in growth factor signaling, growth factor receptors and ligands, and extracellular peptidases (Figure 2, C—E, and Supplemental Figure 1). Areg, a ligand for the Egfr required for epithelial cell proliferation, terminal end bud formation, and ductal elongation in the mouse mammary gland and a known target of ERα action (Ref. 39; reviewed in Ref. 40), was up-regulated 17-fold. Additionally, Egfr, the receptor for Areg, was up-regulated 4-fold by E_2 in the *Ccnd1*$^{+/+}$ but not in the *Ccnd1*$^{-/-}$ mouse mammary gland.

The finding that most E_2-dependent genes required cyclin D1 for their expression in vivo led us to examine further the requirement for cyclin D1 in E_2 signaling in human breast cancer cells. In order to characterize the role of cyclin D1 in estrogen signaling in MCF7 cells, experiments were first performed to determine cyclin D1 responsiveness to E_2 treatment. The effect of E_2 on cyclin D1 mRNA stability has been explored previously (41). E_2 induced the expression of *cyclin D1* at approximately 2 hours with the sequential phosphorylation of Rb (Supplemental Figure 3). E_2 addition to MCF7 cells did not affect cdk4 abundance, but, consistent with prior observations (42), was associated with a modest reduction in the relative abundance of ERα (Supplemental Figure 3A). Cyclin D1 mRNA abundance was induced within 30 minutes (Supplemental Figure 3B), and cyclin D1 promoter activity was induced, consistent with our prior studies (21), demonstrating the cyclin D1 promoter is a transcriptional target of the ERα (Supplemental Figure 3C). These findings are also consistent with prior ChIP analysis of the cyclin D1 gene that identified an ERα binding-enhanced element within the proximal 60 bp of the transcriptional start site (43). E_2-dependent induction of cyclin D1 was monitored after the inhibition of transcription through the use of cycloheximide (Supplemental Figure 3, D and E). Cyclin D1 half-life was not affected by the addition of E_2.

Next we conducted microarray analysis on E_2-stimulated MCF7 cells treated with cyclin D1 siRNA in order to compare the genes regulated by cyclin D1 in mammary gland to those regulated by cyclin D1 in transformed epithelial cells. Hormone-deprived MCF7 cells were treated with cyclin D1 siRNA or control siRNA and stimulated with E_2 or vehicle. In the control siRNA-treated MCF7 cells 2902 genes were differentially regulated by E_2 (Supplemental Figure 4, A and B, and Supplemental Table 3). In contrast, only 244 genes were differentially regulated by E_2 in cyclin D1 siRNA-treated MCF7 cells. Pathway analysis on the 2744 cyclin D1-dependent E_2 -regulated genes showed significant overlap to those pathways previously identified as being mediated by ERα receptor signaling including, cell cycle, mitosis, and DNA repair (Supplemental Figure 4C). These results suggest that the vast majority of E_2-regulated gene expression is dependent upon endogenous cyclin D1 in vivo and in vitro. However, we observe a substantial difference in the genes and pathways that are regulated by cyclin D1 in transformed epithelial cells compared with the whole mammary gland (Supplemental Figure 4D). The difference may be due to distinguishable roles for cyclin D1 in estrogen-stimulated function in transformed epithelial cells vs normal mammary gland.

Whole-genome analysis of cyclin D1 binding in chromatin shows a high incidence of occupancy at genes that are regulated by E_2

A substantial amount of evidence that directly implicates cyclin D1 in regulating transcription through binding DNA sequences within local chromatin of target gene promoters has accumulated over the past decade (29, 44–46). We were interested in comparing genes regulated by E_2 in vivo to those occupied by cyclin D1. Recent studies using ChIP-on-ChIP interrogated approximately 12 000 gene proximal promoter-regulatory regions. Because ERα-responsive elements may be located at distal sites, we extended the analysis to include the additional approximately 99% of the genome using ChIP-Seq technol-

ogy, which provides an unbiased whole-genome approach. We analyzed ChIP-Seq data derived from mouse embryonic fibroblasts derived from *Ccnd1*$^{-/-}$ mice rescued with an expression vector encoding FLAG-cyclin D1 and immunoprecipitated cyclin D1-bound chromatin with an anti-FLAG antibody (36). The anti-FLAG IP approach was used due to the technical challenges of ChIP to endogenous murine cyclin D1 (19, 44, 45). Cyclin D1 was associated with genomic intervals that neighbored approximately 2800 genes (+10 000 to −10 000 of the transcriptional start and end sites, respectively) (36). We then compared the genes occupied by cyclin D1 at the DNA level with those regulated by E_2 in vivo. Approximately 16% of the E_2-regulated genes bound cyclin D1 in ChIP-Seq (Figure 3A and Supplemental Table 2).

The vast majority (88%) of the E_2-regulated genes in the mammary gland in vivo were regulated by E_2 in a cyclin D1-dependent manner (Figure 3B and Supplemental Table 1). The cyclin D1 occupancy of the genes regulated by E_2 in a cyclin D1-dependent manner was distributed upstream of the transcription start site (27 intervals), downstream of the gene (22 intervals) or intragenic (56 intervals) (Figure 3C). Representative tag-density profiles of genes bound by cyclin D1 are depicted in Figure 3D, including *Epgn* (epithelial mitogen homolog), *Eps8* (epithelial growth factor receptor pathway substrate 8), and *Mmp2* (mitotic metallopeptidase 2). A full list of the genes is provided in Supplemental Table 2. The results are consistent with a role for cyclin D1 in an ERα transcriptional program that governs growth factor signaling and peptidases involved in ECM degradation.

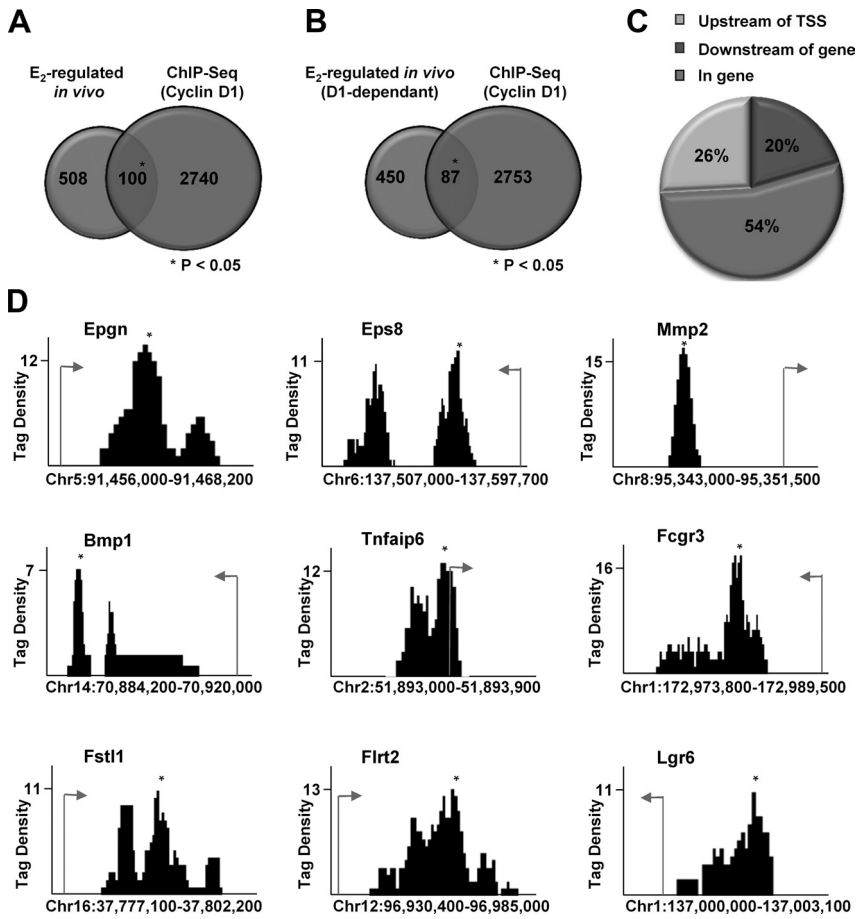

Figure 3. Genes Regulated by E_2 in Vivo Are Bound by Cyclin D1 by ChIP-Seq. A, Comparison of genes occupied by cyclin D1 in ChIP-Seq to those regulated by E_2 in *cyclin D1*$^{+/+}$ mouse mammary gland and (B) those E_2-responsive genes that are regulated in a cyclin D1-dependant manner. C, Location of and (D) representative tag density profiles for, cyclin D1-occupied genes that are regulated by E_2. Vertical axis shows average peak height and horizontal axis depicts chromosomal location of cyclin D1-associated interval sequence. TSS, transcription start site.

Cyclin D1 and BRCA1 converge to regulate *AREG* expression

AREG, a well-known ERα target gene (39), was induced approximately 17-fold by E_2 in the microarray analysis of the *Ccnd1*$^{+/+}$ mammary gland but not in the *Ccnd1*$^{-/-}$ mammary gland (Supplemental Table 1). In order to determine the role of cyclin D1 in E_2-regulated AREG expression, cyclin D1 mRNA was knocked down in MCF7 human breast cancer cells using 3 distinct cyclin D1-targeted siRNA (Figure 4A). AREG expression was measured by Western blot, ELISA, and quantitative RT-PCR (Figure 4, B–D). In MCF7 cells with cyclin D1 siRNA knockdown, we observed a reduction in E_2-induced *AREG* mRNA levels determined by RT-PCR analysis (Figure 4B), a reduction in E_2-induced AREG abundance by Western blotting (Figure 4C), and a reduction in E_2-induced protein by ELISA (Figure 4D).

AREG promoter reporter assays were conducted to determine whether cyclin D1 directly induced *AREG* transcription. Our previous studies had shown that cyclin D1 regulated estrogen-dependent gene expression via a cyclin D1/BRCA1 complex (29). We therefore investigated the possibility that cyclin D1-ERα signaling and BRCA1 signaling may converge on common DNA *cis*-elements of growth factor genes. We deployed an *AREG* promoter fragment known to include the BRCA1 repression element (31). Cyclin D1 expression induced the transcriptional activity of the *AREG* promoter (Figure 4E). Using a series of

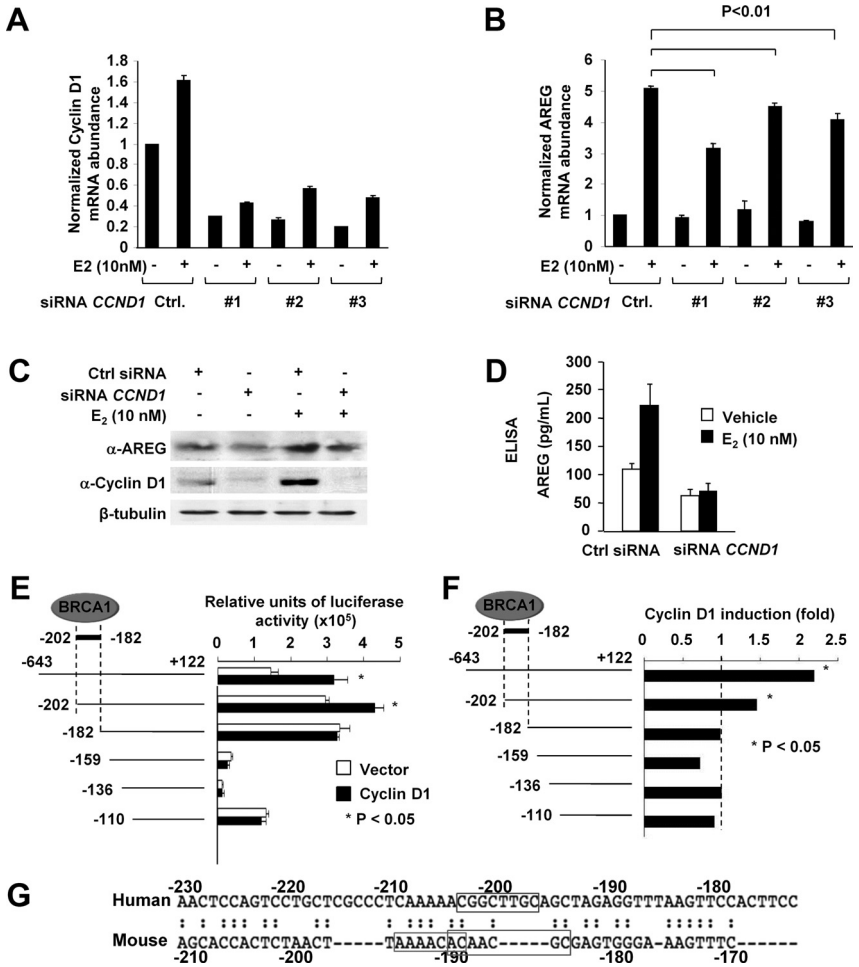

Figure 4. ERα Induces *AREG* Gene Expression in a Cyclin D1-Dependent Manner. A and B, MCF7 cells were transfected with siRNAs targeting cyclin D1 and treated with vehicle or E_2 (10^{-8} M) for 24 hours. *CCND1* and *AREG* mRNA abundance was determined by quantitative RT-PCR. C, Western blot was performed to determine the cellular levels of AREG expression in cyclin D1 knockdown cells compared with scramble siRNA control. β-tubulin was included as loading control for protein abundance. D, The concentration of AREG in cell culture medium was measured by ELISA. Concentration of AREG in the conditioned media was normalized to total protein. Data are mean ± SEM. E, *AREG* promoter luciferase reporter plasmids were transfected into MCF7 cells with a cyclin D1 expression vector. Relative luciferase activity is shown as mean ± SEM normalized to β-galactosidase activity of a cotransfected vector and as (F) fold-induction by cyclin D1. G, Promoter sequence alignment of mouse and human amphiregulin promoter. Homologous nucleotides (:) and regions of discontinuity (−) are indicated. Predicted BRCA1 sites are highlighted for human (− strand), and mouse (2 sites on + strand) with predicted confidence values of 86%, 87%, and 94% respectively. Ctrl., control.

AREG promoter reporter constructs, we identified a region of the *AREG* gene promoter that was responsible for induction by cyclin D1 between −202 and −182 (Figure 4, E and F). This region was previously identified as the BRCA1-repression element of the *AREG* promoter (31). The BRCA1 response element is predicted to also occur in the murine *AREG* promoter with high confidence (87% and 94%) at a similar location (Figure 4G) using a transcription factor prediction software (JASPAR).

To confirm whether BRCA1 and/or the ERα bound to the *AREG* promoter region in local chromatin, ChIP assays were conducted in MCF7 cells. The −3322 to −3169 region was used as a form of negative control. BRCA1 was recruited to both the previously defined ERE of the *AREG* promoter between −912 and −763, and also bound the −202 to −182 region defined as the BRCA1 response elements. ERα was identified by ChIP at both the ERE and BRCA1 response elements but was not identified at the negative control −3322 to −3169 site (Figure 5A). As a form of positive control for ERα, the *pS2* gene was used. ERα was recruited to the *AREG* gene promoter independent of ligand presence although the ERα occupancy of *pS2* gene promoter was entirely dependent on E_2 (Figure 5B). MCF7 cells were transduced with a retrovirus encoding *cyclin D1*; immunoprecipitation-Western blot detected the FLAG epitope of the cyclin D1 protein (Figure 5, C and D). Cyclin D1, detected by anti-FLAG antibody, was recruited to the *AREG* promoter with increased recruitment in the presence of E_2 by FLAG-ChIP (Figure 5D). In order to determine whether the *AREG* gene served as a direct target induced by cyclin D1, ChIP assays were performed using antibodies directed to either FLAG-tagged cyclin D1 or to endogenous cyclin D1. Antibody directed to endogenous cyclin D1 in control MCF7 cells was used to identify cyclin D1 at the *AREG* promoter (Figure 5E). E_2 treatment enhanced recruitment of cyclin D1 to the *AREG* promoter −202 to −182 region. E_2 treatment reduced BRCA1 and ERα occupancy (Figure 5E). Together, these studies demonstrate E_2 treatment results in increased cyclin D1 and reduced BRCA1 occupancy at the *AREG* promoter.

Previous studies demonstrated that immunoprecipitation of BRCA1 coprecipitates cyclin D1 (29, 47). In view of the finding that cyclin D1 regulated the *AREG* promoter via the BRCA1-binding site, we sought to define minimal elements of interaction between cyclin D1 and BRCA1. Previous studies had shown full-length cyclin D1 bound BRCA1 in immunoprecipitation-Western blot and colocalized by confocal microscopy in a subset of nuclear

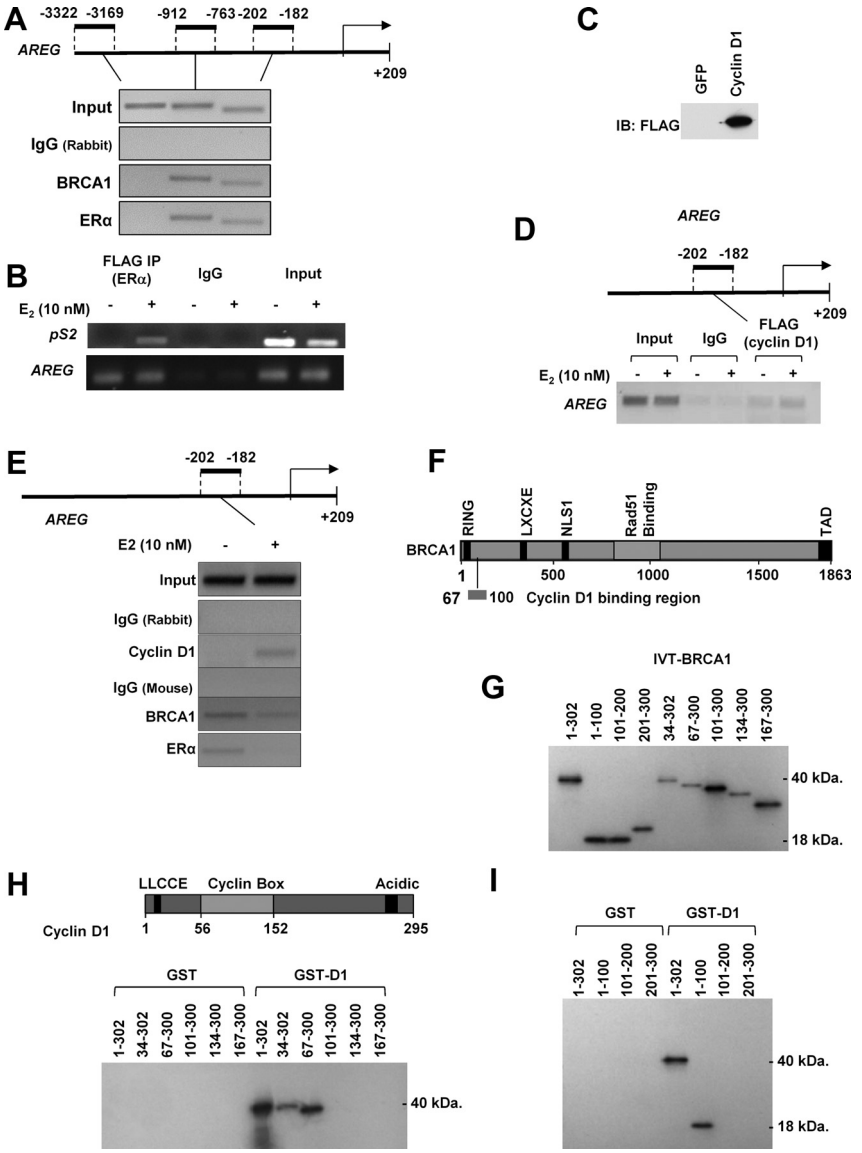

Figure 5. Cyclin D1 Is Recruited to a BRCA1 Binding Site. A, ChIP assay in MCF7 cells treated with E_2 (10 nM) for ERα at the *AREG* gene promoter. B. The *pS2* gene was included as a positive control. C, Western blot shows FLAG-cyclin D1 expression in transduced MCF7 cells. D, ChIP assay to determine cyclin D1 occupancy at the *AREG* gene promoter. Using either MCF7 cells transduced with FLAG-cyclin D1 or (E) MCF7 cells ChIP analysis with antibodies directed to endogenous cyclin D1, BRCA1 or ERα (E_2 10 nM 24 hours). F and G, GST pulldown was performed to determine the minimal region of BRCA1 required for cyclin D1 binding. H and I, GST-cyclin D1 or mutants were incubated with in vitro translated BRCA1. The N terminus (1–100 amino acids) of cyclin D1 was required for BRCA1 binding. IB, immunoblot; IP, immunoprecipitation; IVT, in vitro translation.

dots (29). To define the minimal interaction domains, glutathione-*S*-transferase (GST) pull-down experiments were conducted with GST-cyclin D1 and BRCA1 protein produced by in vitro translation (Figure 5, F and G). The in vitro translated BRCA1 regions between 1–302, 34–302 or 67–300 were sufficient for binding to GST-cyclin D1 (Figure 5H). The GST-cyclin D1 fragment used in pull-down analysis with BRCA1 demonstrated that the BRCA1 region between 1–302 and from 1–100 was able to bind cyclin D1 (Figure 5, H and I).

E_2 increases the abundance of cyclin D1/amplified in breast cancer 1 (AIB1) lower molecular weight coeluting fractions

ERα is known to reside in distinct multiprotein complexes that can be assessed through Superose 6 fractionation of cell lysates. We conducted experiments in order to address whether cyclin D1 may contribute to the formation of the ERα multiprotein complexes. To do so, we first examined MCF7 cells in response to E_2. E_2 treatment resulted in the increased relative abundance of ERα within a high molecular weight complex of approximately 4 mDa (named as HMW complex [HMC]) (Figure 6A). Cyclin D1, which binds ERα in vitro, coeluted in several fractions; in the HMC with BRCA1 and ERα (Figure 6A, fractions 19–22), and cyclin D1 was also observed in 670 kDa (LMW complex [LMC]) complexes (Figure 6A, fractions 31–36). Cdk4 eluted primarily in the 440 kDa complexes (fractions 37–40).

In order to characterize further the proteins within the 670 kDa LMC complexes, Western blot analysis was conducted on the Superose 6 fractions. We examined the effect of E_2 on the distribution of several ERα coactivator known to bind cyclin D1, including SRC-1, AIB1, and P/CAF. E_2 treatment increased abundance of the ERα coactivator AIB1 in the low molecular weight (LMW) fraction of MCF7 cells, with no significant change for SRC1 and P/CAF (Figure 6B). In order to determine whether the coeluting fraction of ERα and AIB1 were physically associated, and to determine whether E_2 increases the amount of AIB1 bound to ERα as previously shown, immune-precipitation Western blotting was conducted (Figure 6C). The addition of E_2 increased the relative abundance of AIB1 associated with ERα. A reduction in cyclin D1 abundance by short hairpin RNA reduced the amount of cyclin D1 associated with ERα but did not abolish AIB1 binding.

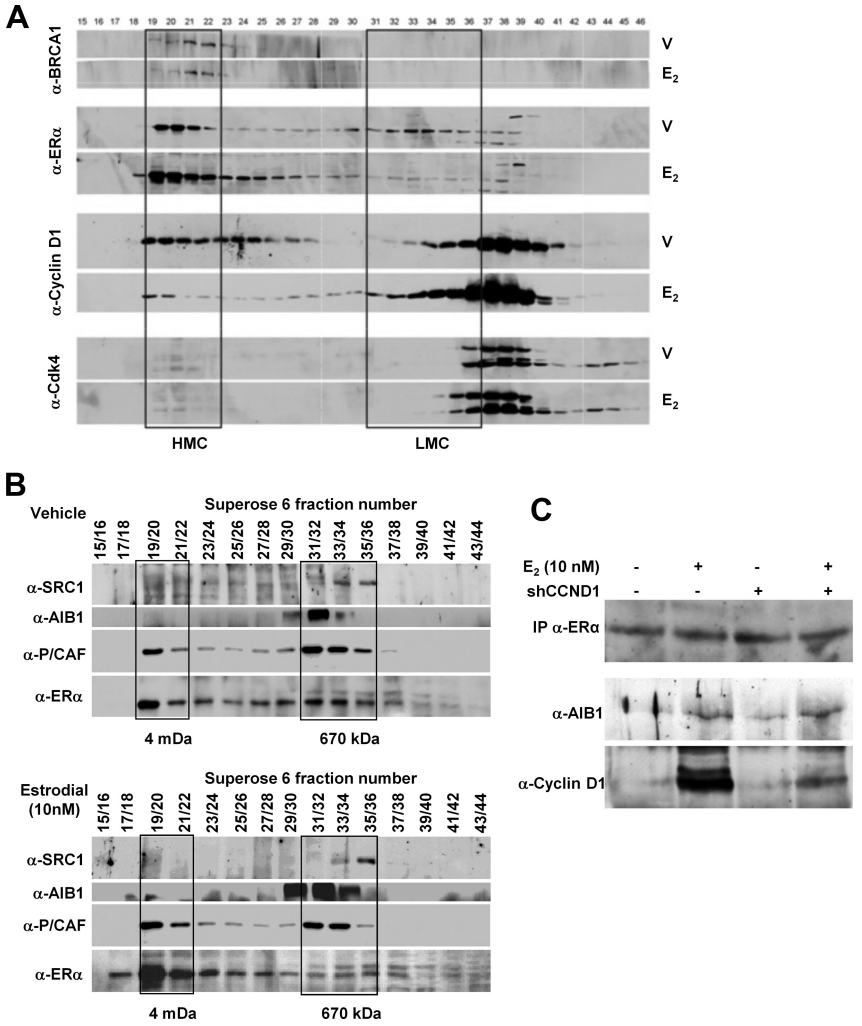

Figure 6. E_2 Induces Cyclin D1 Distribution within a LMW Complex with AIB1. A,. Western blot analysis of Superose 6 chromatography from asynchronously cycling MCF7 cell lysates using antibodies as indicated to the left of the figure. The molecular weight of the fractions is indicated at the bottom of the figure. Cells were treated with (+) E_2 (10^{-8} M). The coeluting fractions > 4 mDa (HMC) and 670 kDa (LMC) are indicated by the boxes. B, Western blot analysis of MCF7 cell extracts after Superose 6 chromatographic fractionation. The antibodies are as indicated. Extracts were treated with E_2 (10^{-8}M) for 30 minutes. Molecular weight markers are shown below panel B, indicating the HMC (4 MDa) or LMC (670 kDa). C, Hormone-deprived MCF7 cells infected with shCCND1 or shControl were treated for 1 hour with 10 nM E_2 or vehicle control; ERα was immunoprecipitated followed by Western blotting for AIB1, cyclin D1, and ERα. IP, immunoprecipitation; V, vehicle.

Cyclin D1 facilitates loading of the ERα coactivator LMW complex (LMC) in vivo

To determine whether cyclin D1 was required for the formation of the 670 kDa LMC complex in epithelial cells expressing BRCA1 and ERα in vivo, analysis was performed on tissues from female *Ccnd1*$^{-/-}$ mice with comparison to age and gender-matched littermate *Ccnd1*$^{+/+}$ control animals. The mammary gland of the *Ccnd1*$^{-/-}$ mice is unsuitable for analysis because the organ develops aberrantly. In contrast, *Ccnd1*$^{-/-}$ liver development is normal, the liver expresses BRCA1, and a substantial literature on ERα-regulated gene expression is documented for this organ. Superose 6 fractionation demonstrated the presence of the 670 kDa LMC complex containing ERα and cyclin D1 in the wild-type mice liver extracts (Figure 7A). In contrast, the ERα from *Ccnd1*$^{-/-}$ cells eluted in the > 4 mDa HMC complex (Figure 7B). These studies demonstrate that cyclin D1 is required for the formation of the 670 kDa LMC ERα complex in vivo.

Discussion

Cyclin D1 is known to regulate gene expression in cultured cells through several different mechanisms (48), indirectly through phosphorylation of pRb and the induction of E2F-dependent gene expression (3) and directly through binding target genes in the context of local chromatin (29, 44, 45). The current studies extend these findings by demonstrating the importance of endogenous cyclin D1 in estrogen-dependent gene expression in vivo. E_2-regulated gene expression (88% [532/608]) was compromised in the mammary gland of *Ccnd1*$^{-/-}$ mice. Cyclin D1 was required for estrogen-dependent expression of a subset of genes. These genes were involved in growth factor and cytokine signaling. We demonstrated two mechanisms by which cyclin D1 augments ERα signaling. First, we demonstrated the requirement for cyclin D1 in the formation of an AIB1/ERα LMW coactivator complex in vivo using *Ccnd1*$^{-/-}$ mice tissue. Second, genome-wide ChIP-Seq analysis identified cyclin D1 occupancy at 16% of cyclin D1-dependent E_2-regulated genes. Further ChIP analysis demonstrated that cyclin D1 is recruited to the BRCA1-binding site of the *AREG* promoter upon E_2 treatment, associated with reduced occupancy by BRCA1. E_2 regulates gene expression through canonical and noncanonical binding sites. The finding that cyclin D1 was identified by ChIP-Seq in ERα-negative cells at 16% of the genes regulated by E_2 in the mammary gland, suggests that cyclin D1 occupies noncanonical as well as

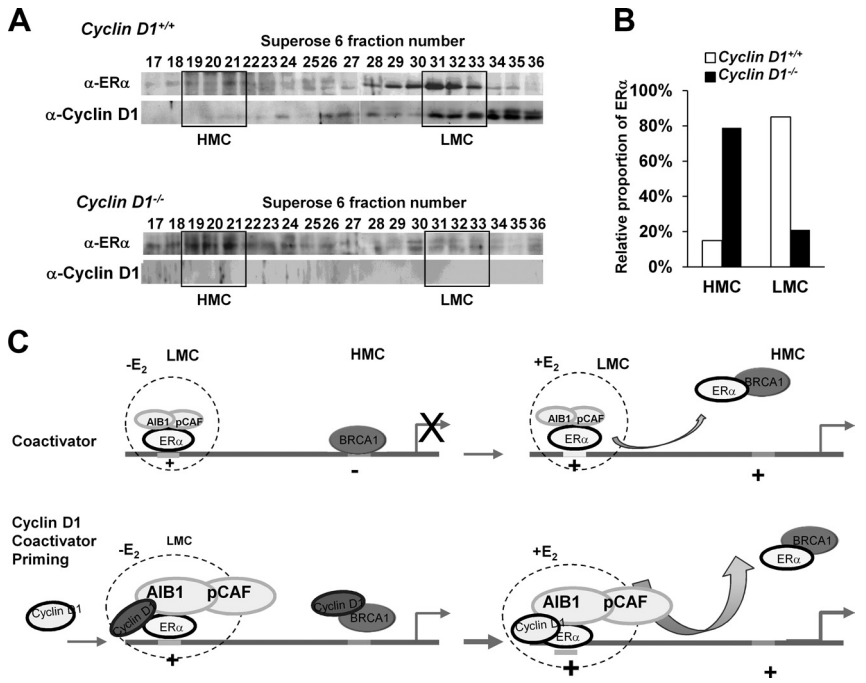

Figure 7. ERα Recruitment to the LMW Complex Requires Cyclin D1. A, Western blot analysis of superose 6 chromatographic fractions from female *cyclin D1*$^{-/-}$ or *cyclin D1*$^{+/+}$ mice cell lysates (liver) using antibodies as indicated to the left of the figure. The molecular weight markers are shown below. B, The relative abundance of ERα in the HMC or LMC (670 kDa) is shown graphically indicating increased ERα in the HMC in *cyclin D1*$^{-/-}$ mice. C, Schematic presentation of cyclin D1 regulation of ERα activation proposes a model in which cyclin D1 participates in ERα signaling by binding to BRCA1 and in the presence of E_2 facilitates an LMC that includes ERα and AIB1. Cyclin D1 binding to BRCA1 antagonized BRCA1 action, including BRCA1 repression of Areg expression.

canonical EREs. However, the chromatin and epigenetic landscapes in mouse embryonic fibroblasts compared with transformed epithelial cells are likely quite different and therefore the cyclin D1-associated transcription factors are likely different. Future studies conducted to survey cyclin D1 in the context of chromatin under hormonal activation in epithelial cells would add to our understanding of this proto-oncogene.

Prior studies demonstrated that progesterone receptor (PR) expression is induced in response to an injection of E_2 in *Ccnd1*$^{-/-}$ mice (49). In contrast, recent studies of mouse mammary tumor virus-cyclin D1 transgenic mice showed enhanced PR expression upon E_2 treatment. The *PR* gene was also induced by cyclin D1 expression in transient expression experiments (50). The current studies extend these prior observations by carefully quantifying the gene expression induced by E_2 in a cyclin D1-dependent manner and by conducting a genome-wide interrogation of candidate target genes. Herein, the induction of *PR* expression by E_2 was reduced 4-fold in the *Ccnd1*$^{-/-}$ mammary gland in vivo; this suggests that cyclin D1 plays a role in augmenting, but is not absolutely required for, E_2 signaling to the *PR* gene.

Growth factors induced by E_2 in a cyclin D1-dependent manner include Areg, which is known to play a critical role in mammary gland development. AREG is a ligand for the epidermal growth factor receptor (EGFR) and is the most abundant growth factor in the pubertal mammary gland (40). The *Areg*$^{-/-}$ mice demonstrated that Areg is essential for breast ductal elongation and estrogen-induced terminal end bud development, which resembles that of the *Esr1* and *Egfr* knockout mice (39). In the current studies, the induction of *Areg* expression by E_2 was abrogated in the *Ccnd1*$^{-/-}$ mammary gland. Endogenous cyclin D1 contributed to a 17-fold increase in E_2-mediated *Areg* expression. Breast epithelial cell proliferation, migration, and invasion are enhanced by AREG. There is a correlation between AREG expression and breast cancer aggressiveness in cells, and the inhibition of AREG expression reduces tumor formation in vivo (51). The 17-fold induction of *Areg* in the murine mammary gland by E_2 may have been enhanced through heterotypic signals. In human MCF7 cells, however, siRNA-mediated reduction of cyclin D1 reduced E_2-induced AREG protein levels, suggesting a cell autonomous effect of cyclin D1 to enhance AREG abundance.

Prior studies using ChIP assays and bioinformatics analysis of ChIP-Seq data demonstrated cyclin D1 occupancy at endogenous EREs in ERα-positive breast cancer cell lines and ERα-negative fibroblasts (29, 36). In these prior studies conducted in ERα expressing cells, E_2 treatment (1 hour) enhanced cyclin D1 recruitment in chromatin to canonical EREs. Herein, expression of cyclin D1 enhanced AREG promoter activity via promoter elements −202 to −182. This element was previously identified as the *AREG* promoter element repressed by BRCA1 (31) and is conserved with the murine *Areg* promoter. Herein we have shown that this region of the AREG promoter binds BRCA1 and ERα. These findings are consistent with prior studies in which cyclin D1 antagonized BRCA1 activity and cyclin D1 was shown to displace BRCA1 occupancy at endogenous target genes in ChIP assays, potentially via phosphorylation of BRCA1 at Ser632 (29, 47).

Knockdown of cyclin D1 in MCF7 cells reduced the E_2-induced secretion of mature AREG into the cell culture medium. This finding suggests that cyclin D1 may function to promote posttranslational modification of AREG. In human breast carcinomas, AREG was overexpressed in half of the specimens whereas staining for AREG in normal mammary gland is only weakly positive (52). The metalloprotease, TNFα-converting enzyme (ADAM17), enhances the shedding of AREG, which is also enhanced by TGFα, HB-EGF, and other ADAM proteins (reviewed in Ref. 53). In the current studies, endogenous cyclin D1 enhanced the abundance of ADAM17, raising the possibility that cyclin D1 may induce AREG maturation/activation through up-regulating ADAM17 expression.

Herein, cyclin D1 enhanced estrogen-dependent expression of several peptidases, in vivo, including Adamts1. In breast cancer cells in culture, estrogen induces patterns of gene expression governing cellular proliferation, migration, and invasion (54). In the current studies of MCF7 cells, distinct gene expression profiles were induced by E_2 compared with the genes induced in the mammary gland in vivo; however, as with the in vivo setting, a substantial proportion of E_2-responsive genes were dependent upon endogenous cyclin D1. The findings are consistent with prior studies in which distinct gene profiles were induced by progesterone in normal vs malignant breast cancer cells (55). The invasion of cells requires proteolytic cleavage of the ECM. The metalloproteinases convey critical functions in cellular migration (24, 49). The disintegrin and metalloproteinase with thrombospondin motif (ADAMTS1) protein bind and degrade the ECM, including aggrecan and versican, to thereby promote pulmonary (56) and bone metastasis (57). Elevated ADAMSTS1 is enriched in metastatic breast cancer (58), and ADAMTS1 promotes primary metastasis of TA3 mammary carcinoma (59). Higher levels of ADAMTS1 are seen in the primary tumors of patients who subsequently develop bone metastases (57). The finding that endogenous cyclin D1 activates matrix peptidases, which in turn can contribute to growth factor signaling, is consistent with several recent studies. Cyclin D1 induced plasminogen activator inhibitor and u-plasminogen activator activity (60), MMP, and Adamts peptidases (Adamts1, Adamts4, Adamts15, Adamts19). The Notch receptor is activated and cleaved by TNFα-converting enzyme (ADAM17). Cyclin D1 was previously shown to induce Notch receptor activity (61). Notch intracellular domain abundance correlates with cyclin D1 abundance in a variety of cell types and cyclin D1 induced notch intracellular domain and enhanced Notch activity (61). The importance of cyclin D1-dependent induction of matrix peptidases in governing tumor progression warrants further analysis.

These studies also extend prior findings that cyclin D1 antagonizes BRCA1 in cultured cells first by showing cyclin D1 induces AREG via the BRCA1 repression element, second by showing the abundance of cyclin D1 is a critical determinant of ERα containing coactivator or corepressor complexes which coeluted with BRCA1, and third by demonstrating that the N-terminal residues 1–101 of BRCA1 are required for interaction with cyclin D1. The finding that BRCA1 amino acids 1–100 bind cyclin D1 supports our prior studies at high resolution in which cyclin D1 associated with BRCA1 via the N-terminal region amino acids 1–500 (29, 47).

Previous studies demonstrated that cyclin D1 interacts with ERα in a ligand-independent manner, and cyclin D1 is known to participate in the formation of an ERα coactivator complex in vitro (22–24). The Superose 6 fractionation studies conducted of murine tissues herein are consistent with the prior findings in tissue culture (22) that cyclin D1 functions as a chaperone protein to facilitate the formation of multiprotein complexes including AIB1 in vivo. In the current studies using mice tissue, the relative abundance of ERα within the LMC was increased in the presence of cyclin D1 (Figure 7C). Deletion of the *Ccnd1*$^{-/-}$ gene resulted in a relative increase in the abundance of ERα in the HMC. Together these studies provide in vivo support for a model in which cyclin D1 facilitates the formation of the ERα-coactivator complex and displaces the ERα corepressor BRCA1. This priming of the ERα coactivator and corepressor complexes serves to coordinate expression of a substantial proportion of ERα-regulated genes in vivo and in tissue culture.

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