Published in final edited form as:
Cancer Res. 2008 September 1; 68(17): 7015–7024. doi:10.1158/0008-5472.CAN-08-0814.

HEXIM1 regulates E_2/ERα-mediated expression of Cyclin D1 in mammary cells via modulation of P-TEFb

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Abstract

Estrogen receptor α (ERα) plays a key role in mammary gland development and is implicated in breast cancer through the transcriptional regulation of genes linked to proliferation and apoptosis. We previously reported that hexamethylene bisacetamide inducible protein 1 (HEXIM1) inhibits the activity of ligand-bound ERα and bridges a functional interaction between ERα and positive transcription elongation factor b (P-TEFb). To examine the consequences of a functional HEXIM1-ERα-P-TEFb interaction *in vivo*, we generated MMTV/HEXIM1 mice that exhibit mammary epithelial-specific and doxycycline-inducible expression of HEXIM1. Increased HEXIM1 expression in the mammary gland decreased estrogen-driven ductal morphogenesis and inhibited the expression of cyclin D1 and serine 2 phosphorylated RNA polymerase II (S2P RNAP II). In addition, increased HEXIM1 expression in MCF-7 cells led to a decrease in estrogen-induced cyclin D1 expression, while downregulation of HEXIM1 expression led to an enhancement of estrogen-induced cyclin D1 expression. Studies on the mechanism of HEXIM1 regulation on estrogen action indicated a decrease in estrogen-stimulated recruitment of ERα, P-TEFb and S2P RNAP II to promoter and coding regions of ERα-responsive genes, *pS2* and *CCND1*, with increased HEXIM1 expression in MCF-7 cells. Notably, increased HEXIM1 expression decreased only estrogen-induced P-TEFb activity. While there have been previous reports on HEXIM1 inhibition of P-TEFb activity, our studies add a new dimension by showing that E_2/ER is an important regulator of the HEXIM1/P-TEFb functional unit in breast cells. Together, these studies provide novel insight into the role of HEXIM1 and ERα in mammary epithelial cell function.

Introduction

Mammary gland morphogenesis and development requires input from several genetic and epigenetic pathways regulated by hormones and growth factors including estrogens (1,2). Estrogens mediate their actions through estrogen receptors (ERs), ERα, and ERβ, nuclear steroid receptors that classically regulate transcription either by directly binding to estrogen-response elements (EREs) of target genes (3-5) or indirectly via protein-protein interactions with other transcription factors like SP1 or AP-1 (6). In both cases, coregulatory proteins are also recruited to the promoter, and together ERs and these factors elicit changes in mRNA and

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protein levels of ER target genes, and ultimately, a physiological response (4-6). Since estrogen signaling controls the balance of growth and apoptosis in normal breast epithelial cells, a disruption of this balance contributes to abnormal cell growth and can lead to tumorigenesis (4,7). Therefore, it is important to identify and elucidate the mechanism of action of ERs and their coregulators that give better insight into ER-mediated transcriptional regulation (8).

In eukaryotic transcription, the elongation stage is highly regulated and important for the generation of full-length mRNA transcripts (9-11). One of the positive regulators, positive transcription elongation factor b (P-TEFb) has an essential role in RNA polymerase II (RNAP II) transcription elongation (9,10). In many human cell types, the predominant form of P-TEFb consists of cyclin dependent kinase 9 (CDK9) and its regulatory partner, cyclin T1 (11). It phosphorylates and thereby inhibits the activity of negative elongation factors, NELF and DSIF (DRB-sensitivity inducing factor) (9). It also phosphorylates the carboxy-terminal repeat domain (CTD) of the largest subunit of RNAP II (9,10). The RNAP II CTD consists of multiple repeats of the heptapeptide sequence, YSPTSPS, phosphorylated at serine 5 by general transcription factor TFIIH during initiation and at serine 2 by P-TEFb during elongation (9, 10). These phosphorylation events are crucial for effective transition from an abortive to a productive phase of elongation (11,12). P-TEFb is essential for productive HIV-1 transcriptional elongation and several studies have shown that various transcription factors bind to and recruit P-TEFb to specific promoters stimulating elongation (12,13).

In previous studies, we identified an ERα-interacting protein, estrogen down-regulated gene-1 (EDG1) (also known as hexamethylene inducible protein 1 (HEXIM1)), and found that it is an inhibitor of ERα transcription and breast cell growth (14). Additionally, we demonstrated that HEXIM1 expression was lower in human breast tumors when compared to adjacent normal tissue, suggesting a role for HEXIM1 in breast tumorigenesis (14). Concurrent studies identified HEXIM1 as a P-TEFb-interacting factor that also inhibits P-TEFb activity (15,16). Studies have also shown that HEXIM2, a paralog of HEXIM1, has the same inhibitory effect on P-TEFb (17,18). We demonstrated that HEXIM1 modulates a functional interaction between ERα and cyclin T1 in breast epithelial cells, and inhibits the recruitment of ligand-bound ERα (E_2/ERα) to the *pS2* gene promoter (19). We also found that cyclin T1 appeared to be necessary for E_2-induction of cyclin D1 protein expression (19). Cyclin D1 (CCND1) is a D-type cyclin that regulates G1-S cell cycle progression during cell proliferation (20). *CCND1* is also E_2/ERα responsive and is thought to play major roles in mammary gland development and breast cancer (1,21). However, the contribution of E_2/ERα to HEXIM1 action in breast cells is not well defined. In addition, the precise mechanism by which P-TEFb regulates *CCND1* transcription and how this can be linked to mammary gland development and tumorigenesis needs to be further defined.

To address these questions, we developed a transgenic mouse model in our laboratory that is doxycycline-inducible and selectively expresses HEXIM1 in the mammary gland under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter previously described (22). Using this model, we demonstrate that increased HEXIM1 expression decreased E_2-induced CCND1 and serine 2 phosphorylated (S2P) RNAP II expression in the mouse mammary gland. In addition, increasing HEXIM1 expression inhibits E_2-induced recruitment of ERα, P-TEFb and S2P RNAP II to ER-responsive genes, *pS2* and *CCND1* in MCF-7 cells. Finally, we observed that E_2 stimulates P-TEFb activity and that HEXIM1 inhibits only E_2-induced, and not basal, P-TEFb activity. These results elucidate the functional consequences of modulating HEXIM1 expression on E_2/ERα-driven transcription in the mammary gland and its implications for estrogen-dependent breast cancer.

Materials and Methods

Materials and Antibodies

17β-Estradiol (E_2) and CDK9 inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were obtained from Sigma Chemical Co. (St. Louis, MO). Commercially available antibodies used for immunoprecipitation and Western blot analysis are described in the Supplementary section.

Transgenic mice (MMTV/HEXIM1) generation

MMTV-rtTA mice were generated as described by Gunther et al (22). To generate pTET-HEXIM1 mice, a plasmid construct was made by subcloning the coding sequence of human HEXIM1 gene downstream of the tetracycline-dependent minimal promoter in the pTet-splice transgene construct. After purification, the resulting plasmid was used for pronuclear injection into FVB oocytes (Case Western Reserve University Transgenic and Targeting Facility). To achieve mammary gland-specific expression of HEXIM1 in a doxycycline-dependent manner, pTET-HEXIM1 mice were crossed with the MTB line, which expressed the reverse tetracycline-dependent transcriptional activator (rtTA) under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR). From these matings the bigenic mice, MMTV/HEXIM1, were created. Transgene expression was induced by adding 2 mg/ml doxycycline to the drinking water. Bigenic mice were identified by screening genomic DNA from tail biopsies for the presence of the transgenes using PCR and verified by Western blot analysis. See Supplemental Table 1 for list and sequences of primers used.

Whole-mount histology & Immunodetection

Mice were induced with doxycycline at 9 weeks of age, ovariectomized and treated with E_2 at 12 weeks. E_2 was administered as daily subcutaneous injections of sesame oil solution containing 1μg of E_2. Mammary glands from the MMTV/HEXIM1 mice were collected 25 days after start of E_2 treatment for whole-mount staining via the Carmine-alum technique and Western blot analyses. Immunohistochemistry using sections from mammary glands are described in the Supplementary section.

Reverse Transcription (RT) PCR Analyses

Human breast epithelial cells, MCF-7, were maintained as described (19) and were transiently transfected with pCMV-TAG2B or pCMV-TAG2B-HEXIM1 using FuGENE HD reagent (Roche, IN) according to the manufacturer's instructions. Forty-eight hours later, cells were treated with ethanol vehicle or 100 nM E_2 for 3 hours. Total RNA was extracted using the TRIzol reagent (Invitrogen, CA) and subjected to RT-PCR analyses as described in the Supplementary section.

Western Analyses

Western blot analyses using extracts from mammary glands and MCF-7 cells are described in the Supplementary section.

RNA interference

A Pol II promoter-driven miRNA expression vector system (Invitrogen, CA) was used. To make pcDNA-HEXIM1 miR, miRNA oligos (see Supplemental Table 3 for list and sequences) were annealed and cloned into the pcDNA 6.2 GW/EmGFP vector (Invitrogen) according to the manufacturer's instructions. MCF-7 cells were transfected with pcDNA 6.2-GW/EmGFP-miR expression vectors containing either the HEXIM1 miRNA insert or a control LacZ miRNA insert. Following blasticidin selection, cells expressing the highest level of GFP were flow-

sorted and expanded. During experiments, cells were treated with ethanol vehicle or 100 nM E_2 for 3 hours and harvested as described above for Western blot analyses.

Chromatin immunoprecipitation (ChIP) assays

MCF-7 cells were plated on 150 mm plates and transfected as described with pCMV-TAG2B or pCMV-TAG2B-HEXIM1. Forty-eight hours later, cells were treated with ethanol vehicle or 100 nM E_2 for 45 and 90 minutes. ChIP assays were carried out as previously described (19). See Supplemental section for further details of immunoprecipitation and analyses.

CTD kinase assays

Kinase assays were performed according to previously described protocols with some modifications (23,24). See Supplemental section for complete description of assay.

Data analyses

Statistical significance was determined using Student's *t* test comparison for unpaired data and was indicated as follows: *, $P < 0.05$; **, $P < 0.005$.

Results

Increase in HEXIM1 expression inhibits E_2-driven mammary gland development by decreasing cell proliferation and increasing apoptosis

To examine the functional consequences of an interaction between HEXIM1, ERα and P-TEFb in the mammary gland, we generated a double transgenic mouse model, MMTV/HEXIM1, which inducibly overexpress HEXIM1 in the mammary gland when the mice are treated with doxycycline (+DOX). We first examined the effects of elevated levels of HEXIM1 on E_2-driven mammary gland development by having the MMTV/HEXIM1 mice ovariectomized and treated with E_2 as described in “Materials and Methods”. In comparing whole mounts of mammary glands from -DOX and +DOX animal groups, we observed decreased ductal branching in the mammary glands of +DOX mice when compared to -DOX mice, as well as compared to single transgenics (MMTV alone) (Figure 1A, −/+DOX panel insets). We quantified the level of increase of HEXIM1 expression in the mammary gland as approximately 24% over endogenous levels (data not shown, see Figure 2A for immunoblot), so we do not foresee that an overwhelming amount of HEXIM1 is needed to dictate these physiological changes. Since ductal elongation and branching in the mammary gland have been shown to be E_2/ERα-dependent (1, 25), these data suggest that HEXIM1 inhibits E_2/ERα-driven mammary gland morphogenesis.

Previously, we found that HEXIM1 inhibits ERα transcription (14,19), so this physiological effect could be due to a dysregulation of ER-responsive genes involved in proliferation and apoptosis. To investigate this, MMTV/HEXIM1 mice were injected with BrdU two hours prior to being sacrificed. BrdU-labeled nuclei in the mammary gland were detected by immunostaining and apoptotic nuclei were stained by TUNEL. Quantitation of positively-labeled mouse mammary epithelial cell nuclei revealed both a decrease in epithelial cell proliferation and an increase in apoptosis (Figure 1B). Taken together, our data suggest a critical role for HEXIM1 in E_2/ERα-driven processes in the mammary gland.

Increased HEXIM1 expression regulates cyclin D1 protein expression levels and Serine 2 phosphorylation of RNA polymerase II *in vivo*

Our findings that overexpression of HEXIM1 decreased ductal branching and cell proliferation in the mammary gland prompted us to investigate the effect of HEXIM1 overexpression on E_2/ERα signaling. As an output, we examined changes in cyclin D1 (CCND1) and c-Myc

because both genes are E_2/ERα responsive and involved in proliferation integral to mammary gland development and breast cancer (26,27). MMTV/HEXIM1 mice were ovariectomized and treated with E_2 as described and we found that increased HEXIM1 expression (+DOX) resulted in decreased CCND1 protein expression levels (~67%) in mouse mammary gland cell extracts (Figure 2A). However, c-Myc expression levels did not significantly change regardless of HEXIM1 expression (Supplemental Figure 1A), suggesting a difference in sensitivity of ERα target genes to HEXIM1.

In addition, since HEXIM1 inhibits P-TEFb activity (12), we examined the effect of increased HEXIM1 expression on P-TEFb activity in the mouse mammary gland by investigating the expression levels of the serine 2 phosphorylated form of RNAP II (S2P RNAP II). In +DOX mice, we observed a decrease in S2P RNAP II levels (~68%) (Figure 2A). It is important to note here that the antibody used to detect S2P RNAP II recognizes both phosphorylated (RNAP IIo) and unphosphorylated (RNAP IIa) forms of RNAP II. We also blotted with another antibody that detects only the RNAP IIa form (Hypo RNAP II) and found that there was no observable change between −/+DOX mice groups (Figure 2A). Also, HEXIM2, a paralog of HEXIM1, inhibits P-TEFb activity (12), but in the mammary gland, HEXIM1 protein expression levels are significantly higher (Supplemental Figure 1B), suggesting a dominant role for HEXIM1 in the mammary gland.

We also examined the expression levels of the serine 5 phosphorylated form of RNAP II (S5P RNAP II), associated with initiation, using immunofluorescent labeling of epithelial nuclei in mammary gland lumen from MMTV/HEXIM1 mice. We did not observe any changes in the percentage of S5P RNAP II positively-stained nuclei (S5P +ve cells) when we compared −/+DOX animal groups (Figure 2B). However, in the +DOX animal group, the percentage of S2P RNAP II positively-stained nuclei (S2P +ve cells) within the mammary gland lumen was significantly decreased by 50% ($P < 0.05$) when compared to the −DOX group (Figure 2B), verifying our results in the Western blot (Figure 2A). Given that E_2/ERα regulates CCND1 expression (21, 27) and HEXIM1 inhibits P-TEFb activity and associates with ERα (12, 19), the effect of increased HEXIM1 expression levels on CCND1 and S2P RNAP II expression suggest that these changes reflect a complex interaction of multiple pathways that converge at the level of E_2/ERα-mediated transcription.

HEXIM1 regulates cyclin D1 and pS2 expression levels *in vitro*

The effects of increased HEXIM1 expression on CCND1 and S2P RNAP II expression levels could also be the sum result of the disruption of E_2/ERα activity in multiple cell types in the mammary gland. To verify that our observations can be attributed to a more localized event in epithelial cells, we investigated the effects of increased HEXIM1 expression in mammary epithelial MCF-7 cells. MCF-7 cells were transfected with pCMV-Tag2B-HEXIM1 or control vector and treated with ethanol vehicle or E_2 for 3 hours. In control cells, we observed an average 1.5 to 2-fold E_2 induction of both CCND1 mRNA and protein levels (Figures 3A and B); consistent with what has been demonstrated in other studies (8, 21). As expected, increased HEXIM1 expression diminished E_2-induction of CCND1 mRNA and protein expression (Figures 3A and B, compare lanes 2 and 4). For CCND1 protein expression levels, this was quantified as a 52% decrease in expression by HEXIM1 when normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (Figure 3B).

We also investigated the effect of increased HEXIM1 expression on other ER-responsive genes and found increased HEXIM1 levels decreased E_2-induced increases in pS2 mRNA and protein levels (Figures 3A and B, Supplemental Figure 2A). The E_2-induced mRNA levels of other genes including c-Myc, Progesterone receptor (PR) and Cathepsin D remained unchanged with increased HEXIM1 expression (Figure 3A, Supplemental Figure 2A). In addition, E_2-induction of c-Myc protein levels was also unchanged with increased HEXIM1 expression (Figure 3B,

with quantitation in Supplemental Figure 2B). These data also suggest that increased HEXIM1 expression may not have a similar effect on all ERα-target genes.

Having observed that increased HEXIM1 expression decreased E_2-induced CCND1 and pS2 expression, it stood to reason that HEXIM1 silencing would result in an increase in the induction of both genes by E_2 when compared to control cells. HEXIM1 gene expression knockdown (~50%) using miRNA-mediated RNAi resulted in a statistically significant ($P < 0.01$) enhancement of E_2-induced CCND1 protein expression levels (Figure 3C). We did not observe a similar effect with pS2 (data not shown) and E_2-induced c-Myc protein expression levels remained unchanged (Figure 3C). Because multiple regulatory proteins are involved in CCND1 regulation (28), we cannot rule out possible actions of other factors *in vivo*. Also, we cannot rule out the possibility of HEXIM1 regulation of other key E_2 responsive genes involved in cell proliferation and mammary carcinogenesis not studied here. Nonetheless, taken together our data support a novel physiological role for HEXIM1 in E_2-driven mammary gland development via regulation of CCND1 levels and serine 2 phosphorylation of RNAP II.

HEXIM1 inhibits E_2-induced recruitment of ERα and cyclin T1 (P-TEFb) to ERα-responsive genes

Given our observations on the effect of HEXIM1 expression on CCND1 and pS2, it is critical to characterize the molecular events involved in HEXIM1 transcriptional regulation of these ER-responsive genes. We previously reported that HEXIM1 associates with ERα after 90 minutes of E_2 stimulation in MCF-7 cells and increased HEXIM1 expression led to a decrease in E_2/ERα recruitment to the pS2 promoter (19). Other reports have shown that P-TEFb associates with the elongating form of RNAP II, S2P RNAP II, which is thought to predominate in the coding regions of genes with increased occupancy towards the 3' end of genes (29-31). Therefore, we hypothesized that increased HEXIM1 levels would (1.) inhibit the recruitment of ERα to the promoter region of ER target genes and (2.) inhibit the recruitment of P-TEFb to regions downstream from the promoter at ER-responsive genes, and this would in turn verify the potential regulation of transcriptional elongation of these genes. To investigate this, we carried out chromatin immunoprecipitation (ChIP) assays in MCF-7 cells and examined the effect of E_2 on ERα and P-TEFb occupancy at two ERα-responsive genes, *pS2* and *CCND1*.

We found that an increase in HEXIM1 expression in MCF-7 cells correlated with a 2-fold increase in HEXIM1 occupancy at E_2-responsive regions within the *pS2* and *CCND1* (32) promoters (Supplemental Figure 3A) that was not significantly affected by E_2 treatment. We also found that increased HEXIM1 expression inhibited the recruitment of E_2/ERα to the promoter regions of *pS2* and *CCND1* (Figure 4B). Now, it is well documented that E_2/ERα cycles on and off the promoter of ER-responsive genes and ERα binding is typically diminished after 90 minutes of E_2 stimulation (33,34). Other studies have also shown that the absolute timing of ERα cycling differs and there can still be significant E_2/ERα enrichment at 90 minutes (35,36). In our studies, it is clear that E_2/ERα binding is less at 90 minutes when compared to 45 minutes (Figure 4B, compare lanes 2 and 3), and we see a significant decrease at 3 hours (data not shown) indicating E_2/ERα is cycling on DNA in our experiments. Regardless of the time point of E_2 stimulation, increased HEXIM1 inhibits E_2/ERα recruitment to DNA.

For P-TEFb (via cyclin T1), we observed an E_2-dependent recruitment pattern that differed slightly at the promoter and coding regions of both *pS2* and *CCND1* genes. At the promoters of both *pS2* and *CCND1*, cyclin T1 follows a similar trajectory as ERα in terms of recruitment in control cells, but there is no significant effect on cyclin T1 recruitment with increased HEXIM1 expression (Figure 4C). However, at the coding regions of both genes, we observed a gradual increase in cyclin T1 recruitment with over time with E_2 stimulation and, increased HEXIM1 expression inhibited the recruitment of cyclin T1 (Figure 4C). As a further control, we looked at cyclin T1 recruitment to the *GAPDH* ORF at a region that has been shown to

have significant RNAP II enrichment during GAPDH transcription (37). We did not observe an E_2-dependent recruitment pattern and there were no significant changes in cyclin T1 recruitment with increased HEXIM1 expression (Supplemental Figure 3B).

These data suggest that HEXIM1 inhibits ERα transcriptional elongation by inhibiting PTEFb recruitment, via cyclin T1, to the coding region of some ER-responsive genes. This result is consistent with other studies showing that the recruitment of P-TEFb to promoter-proximal and coding regions stimulates transcriptional elongation (30,38). Also, in previous studies, we observed a decrease in the co-recruitment of cyclin T1 with ERα at the *pS2* promoter with increased HEXIM1 expression (19), but it appears that the total occupancy of cyclin T1 at the promoter region does not change with increased HEXIM1 expression (Figure 4C). In this context, our data suggests a dual role for P-TEFb in ERα transcription. On one hand, it acts as a coactivator for ERα-driven transcription by directly associating with E_2/ERα (19), but it also serves its general purpose as a transcription elongation factor, with E_2 acting as an enhancer of P-TEFb occupancy in the context of ER-responsive genes.

HEXIM1 inhibits E_2-induced P-TEFb activity and recruitment of serine 2 phosphorylated RNA polymerase II to coding regions of ER-responsive genes

Since E_2 enhanced P-TEFb recruitment to *pS2* and *CCND1* genes, we investigated if E_2 also increased P-TEFb activity as a means of promoting transcriptional elongation of ERα-responsive genes. To do this, we performed kinase assays using endogenous immunoprecipitates of cyclin T1 from MCF-7 cells and examined the kinase activity of P-TEFb with a synthetic peptide substrate, CTD4 ($YSPTSPS_4$). As shown in Figure 5A, E_2 treatment increased P-TEFb activity in MCF-7 cells (compare lanes 2 and 5). We also observed that increased HEXIM1 expression in MCF-7 cells inhibited an E_2-induced increase in P-TEFb activity (Figure 5A, compare lanes 2 and 5 to lanes 8 and 11). We confirmed that this inhibition was selective for P-TEFb by adding the CDK9 inhibitor, DRB, to an equal half of the kinase immunoprecipitates (Figure 5A, lanes 3, 6, 9 and 12) and that the input levels of cyclin T1 and HEXIM1 were evenly loaded (Supplemental Figure 4A). Quantitation of ^{32}P incorporation into the CTD4 peptide verified that HEXIM1 significantly abrogates E_2-induced P-TEFb activity in MCF-7 cells (Supplemental Figure 4B).

We also observed that increased HEXIM1 expression did not inhibit basal P-TEFb activity in HEXIM1-transfected MCF-7 cells compared to non-transfected cells (Figure 5A, compare lanes 2 and 8). One reason for this could be attributed to the fact that at basal levels, the P-TEFb complex is thought to occur in two states, 50% free and 50% HEXIM1-7SK RNA bound (12) and in a study by He et al, gradually decreasing HEXIM1 expression by RNAi did not initially have much effect on this equilibrium because it was the “free” form of HEXIM1 that was being down-regulated, without any effect on the HEXIM1 protein associated with P-TEFb (11). In our experiments, it is possible that increasing HEXIM1 expression may not be significantly perturbing the P-TEFb equilibrium initially, but increases the availability of free HEXIM1 populations that subsequently diminishes any increases in E_2 induced P-TEFb activity.

Since HEXIM1 inhibited the recruitment of cyclin T1 to the coding regions of ER-responsive genes and E_2-induced P-TEFb activity, we determined the effect of increased HEXIM1 levels on the recruitment of S2P RNAP II as a mark of the modulation of transcription elongation (9). At the *pS2* promoter, we found that E_2 stimulated the enrichment of all forms of RNAP II, without any significant changes when HEXIM1 expression was increased (Figure 5B). This result differs with what has been observed in some studies, which show low S2P RNAP II occupancy in comparison to S5P RNAP II at the promoter of transcriptionally active genes (29,37). However, it is possible for both S2P and S5P RNAP II to occupy similar regions on DNA (39), which likely marks the beginning of the transition to elongation, but it is unclear

since we did not study proximal upstream or downstream regions to the *pS2* promoter. In addition, there was no change in the recruitment of all forms of RNAP II recruitment to the *CCND1* promoter in both control and HEXIM1-transfected cells, although it was not as sensitive as the *pS2* promoter to E_2 stimulation (Figure 5C).

However, at the *pS2* and *CCND1* coding regions, we found that increased HEXIM1 expression inhibited the E_2-dependent recruitment of S2P RNAP II, without significant changes in the recruitment of S5P and unphosphorylated RNAP II forms (Figures 5B and 5C). These data indicate that in mammary epithelial cells, HEXIM1 does not affect transcription initiation, since the recruitment patterns of all forms of RNAP II to the promoter-regions of *pS2* and *CCND1* were unchanged regardless of HEXIM1 expression levels in the cell. However, increased HEXIM1 decreases transcription elongation since the recruitment of S2P RNAP II to the coding region was decreased.

Discussion

This study provides novel evidence for a physiological role of HEXIM1/P-TEFb interaction in attenuating E_2/ERα driven transcription in the mammary gland and breast cancer cells. First, we demonstrated that increased HEXIM1 expression in the mammary gland of a transgenic mouse model decreased ductal branching, an E_2-driven developmental process, due to changes in proliferation and apoptosis. We correlated these changes with a decrease in CCND1 and S2P RNAP II expression *in vivo* and *in vitro*. We also show that overexpression of HEXIM1 diminished E_2-induced recruitment of ERα and cyclin T1 to the promoter and coding regions, respectively, of ER-responsive genes. Further, we show that E_2 enhances the activity of the P-TEFb kinase, CDK9, which is inhibited by increased HEXIM1 expression. Surprisingly, increased HEXIM1 expression inhibited only E_2-induced increases in P-TEFb activity and not basal P-TEFb kinase activity. In our studies, this inhibition of P-TEFb activity translates to a decrease in the recruitment of S2P RNAP II, and not other forms of RNAP II, to the coding regions of ER-responsive genes, *pS2* and *CCND1*. These findings support a role for P-TEFb and transcription elongation in cell proliferation but more importantly, the data suggests a novel mechanism of action for HEXIM1 that can be recapitulated *in vivo* and a possible therapeutic role for HEXIM1 in hormone-dependent breast cancer.

Given that the regulation of *CCND1* is complex (28), we do not assume that our findings represent a comprehensive explanation regarding E_2-regulation of *CCND1*. Also, other sites within *CCND1* contribute to the transcriptional output (21). We investigated the recruitment patterns of ERα, P-TEFb and RNAP II to two sites within the gene: an E_2-responsive region of the promoter and the coding region. Perhaps, a more extensive analysis of different sites within the genes, *pS2* and *CCND1,* and even other ER-responsive genes will reveal other insight into mechanism of HEXIM1 regulation of these genes. However, we believe that the information gathered from this study was sufficient to demonstrate the regulatory effects of HEXIM1 on ERα and P-TEFb recruitment to *pS2* and *CCND1*, suggesting a role for HEXIM1 and P-TEFb in ERα transcriptional regulation of some, but not all ERα target genes.

Based on our previous (19) and current studies, we speculate that E_2 enhances an ERα-P-TEFb interaction, and this increases the population of active P-TEFb at the gene locus of ER-responsive genes, which in turn phosphorylates the CTD of RNAP II. This phosphorylation event positions the gene in an active elongation state with increased S2P RNAP II occupancy at the coding regions, and enhanced recruitment of other forms of RNAP II marking the gene in an active transcription state (31,39). However, increased HEXIM1 expression inhibits ERα and P-TEFb enrichment at the promoter and coding regions, respectively, of ER-responsive genes thus, decreasing the population of P-TEFb available to phosphorylate RNAP II to the serine 2 phosphorylated form associated with transcriptional elongation. In addition,

we observed that increased HEXIM1 inhibits E_2-induced P-TEFb activity and postulated that this was due to an increase in the "free" form of HEXIM1, which serves to diminish any subsequent increases in PTEFb activity. Taken together, this scenario could represent the mechanism by which HEXIM1 modulates ERα-mediated transcription in the context of some ER-responsive genes (See Figure 6 for proposed model).

The understanding of general mechanisms that control elongation stem from studies showing that HIV-1 harnesses P-TEFb as a cofactor to promote efficient mRNA transcript synthesis (12). These and other studies have raised questions about whether P-TEFb acts as a general transcription elongation factor or serves in a gene-selective or context-dependent manner. The P-TEFb complex components, cyclin T1 and CDK9, have not been shown to have sequence-specific DNA binding activity, but transcription factors interact with and recruit P-TEFb to their respective promoter targets (12,40,41). In addition, DNA microarray analyses of hearts from cyclin T1 transgenic mice indicate selective increases in subsets of genes, rather than a global increase in mRNA expression when compared to non-transgenic mice (42). Our studies suggest that in breast epithelial cells, P-TEFb can be modulated by E_2/ERα and HEXIM1 in the context of some ERα target genes, although identical E_2-induced recruitment patterns for P-TEFb to *pS2* and *CCND1* genes suggest a general transcription elongation mechanism is also involved. In addition, the interaction of P-TEFb and E_2/ERα supports a positive aspect of ERα transcription elongation regulation, but E_2/ERα also interacts with negative elongation factor (NELF) and this interaction inhibits ERα-mediated transcription (43).

HEXIM1 has been shown to have P-TEFb-independent action as seen with the glucocorticoid receptor (44). We have also reported on P-TEFb-independent action of HEXIM1 in cardiovascular development (45). Given this evidence, it is clear that HEXIM1 can inhibit transcription in a P-TEFb-dependent and -independent manner. Therefore, we cannot assume that the effect of HEXIM1 in the mammary gland is solely on ERα/P-TEFb, as other factors are involved in mammary gland development. However, we were able to demonstrate a specific inhibition pattern that HEXIM1 exerted on E_2-induced events at ER-responsive genes, *pS2* and *CCND1*, and increased HEXIM1 levels inhibited E_2-induced P-TEFb activity. Thus, based on our data, the HEXIM1 inhibition patterns observed in this study is largely P-TEFb-dependent in both our cell culture and animal models. Several studies also support an emerging role for HEXIM1 as a regulator of cell growth and differentiation (12,46). Conversely, deletion of CLP-1, the mouse HEXIM1 gene, leads to pathological cardiac hypertrophy and perinatal death (47).

In this study, a targeted increase in HEXIM1 expression in the mouse mammary gland driven by a mammary epithelial cell promoter (MMTV-LTR), led to a decrease in ductal branching (Figure 1A), an E_2/ERα driven mammary gland developmental process (25). This observation was attributed to a decrease in proliferation and an increase in apoptosis (Figure 1B). The decrease in proliferation was linked to a concurrent decrease in CCND1 expression, further demonstrating that HEXIM1 regulates ER-responsive genes *in vivo*. Future studies will not only aim to address HEXIM1 regulation of other E_2/ERα target genes, but also HEXIM1 regulation of other nuclear receptors relevant in mammary cell function and tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to Dr. Huayun Deng for his help with some of the animal work and advice on immunohistochemistry. We thank Dr. Lewis Chodosh (University of Pennsylvania) for the MTB mice and the pTet-Splice vector. This work

was supported by National Institute of Health Grant CA92440 to M.M.M and a Department of Defense Predoctoral Fellowship W81XWH-06-1-0426 to N.O.

Abbreviations

ERα	Estrogen receptor alpha
E_2	17-beta estradiol
HEXIM1	Hexamethylene inducible gene-1
P-TEFb	Positive transcription elongation factor b
CTD	Carboxy-repeat terminal domain
RNAP II	RNA polymerase II
CCND1	cyclin D1

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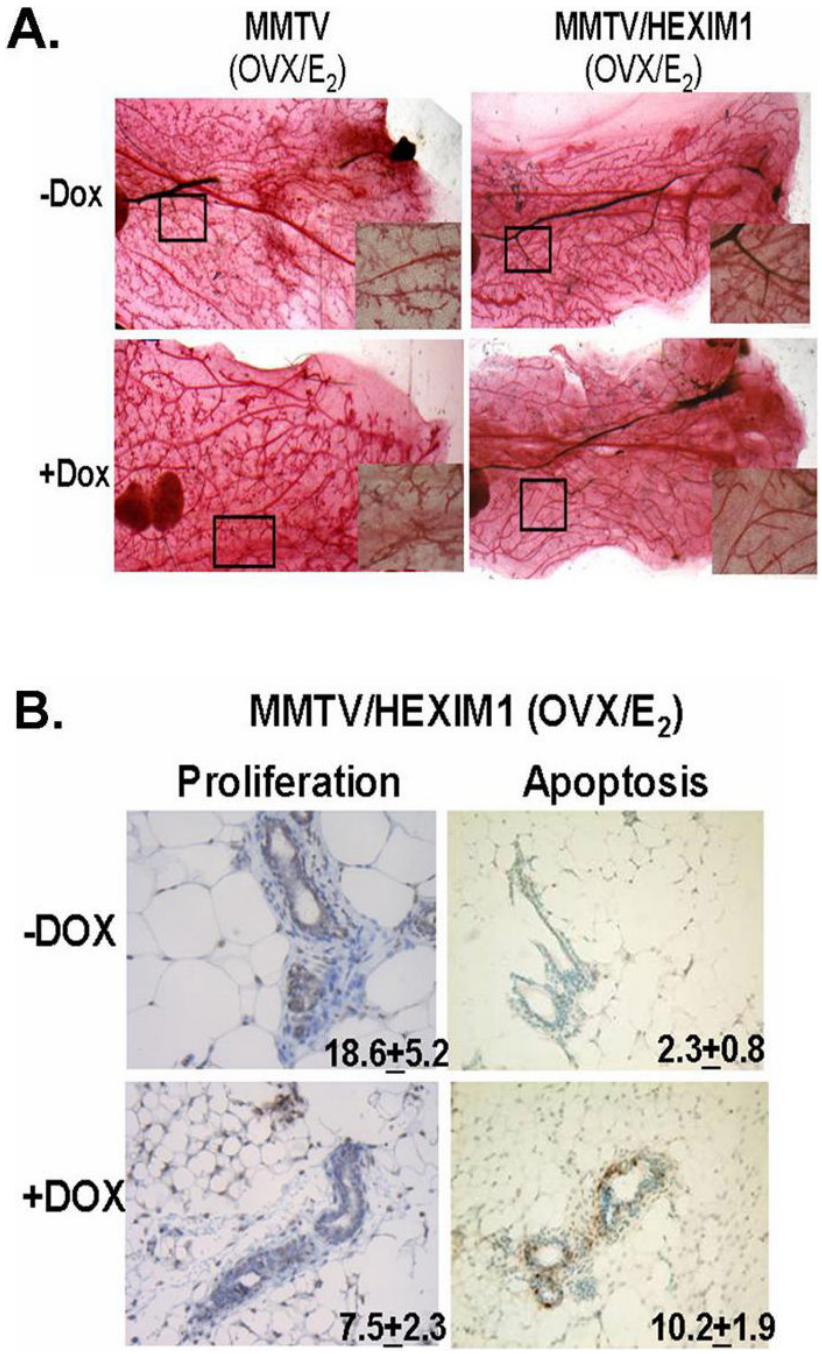

Figure 1. Increased HEXIM1 expression inhibits estrogen-regulated mammary gland morphogenesis due to changes in proliferation and apoptosis

A. MMTV/HEXIM1 mice were treated as described in *Materials and Methods*. Representative whole mounts were obtained from MMTV and MMTV/HEXIM1 mice with Carmine alum stain. Original magnification is X40 for all panels.

B. BrdU-labeled nuclei were detected by immunostaining and apoptotic nuclei were stained by TUNEL. Quantitation of positively-labeled MMEC nuclei from at least 1000 nuclei each from 5 mice per group (−/+DOX) is shown. Original magnification is X40 for all panels.

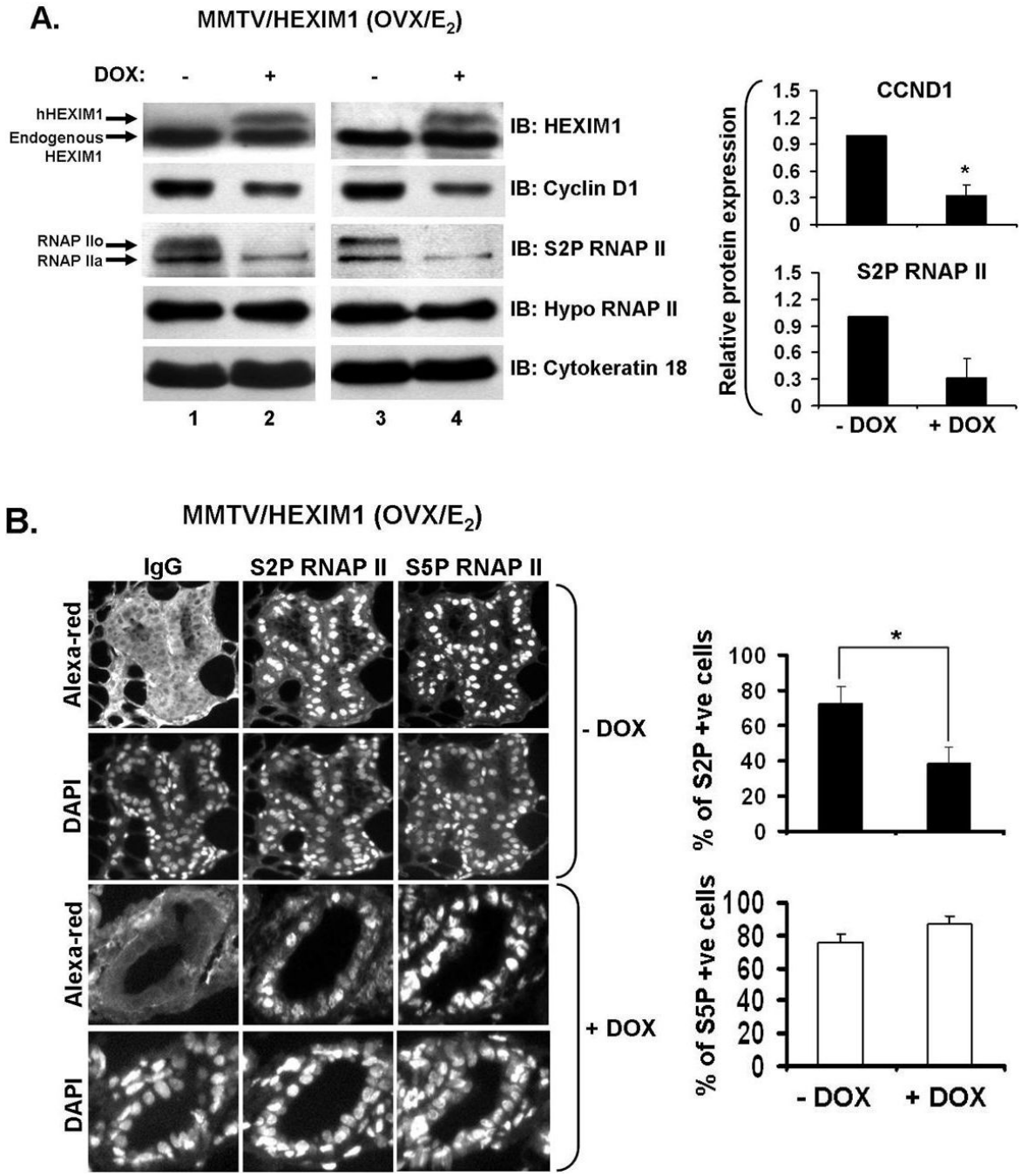

Figure 2. Increased HEXIM1 expression inhibits E_2-induced cyclin D1 expression and serine 2 phosphorylation of RNAP II in mouse mammary gland

A. MMTV/HEXIM1 mice were treated as described in *Materials and Methods*. Mammary gland tissue extracts from MMTV/HEXIM1 mice were subjected to Western blot using indicated antibodies for immunoblotting. Anti-cytokeratin 18 was used as an epithelial cell marker and a loading control. RNAP IIa and IIo indicate the “hypo” and “hyper” phosphorylated forms of RNAP II respectively. Samples were collected from 3-5 mice per group (−/+Dox) and columns represent quantitation of Western analyses for cyclin D1 and S2P RNAP II; *bars*, SE; *, $P< 0.01$.

B. Immunofluorescent detection of S2P and S5P RNAP II. Representative images of mammary gland sections from MMTV/HEXIM1 mice (−/+ Dox) stained as described in *Materials and Methods* with S2P and S5P RNAP II-specific antibodies. Nuclei were counterstained with DAPI as indicated. Original magnification is X20 for all panels. Quantitation of S2P or S5P positively stained (S2P+ or S5P+) luminal epithelial cells was performed as described in Materials and Methods. Columns represent percentage (mean ± SEM) of total S2P/S5P+ luminal epithelial cells with 3-4 mice per group (−/+ Dox) and a minimum of 1000 cells per animal analyzed; *bars*, SE; *, $P<0.05$

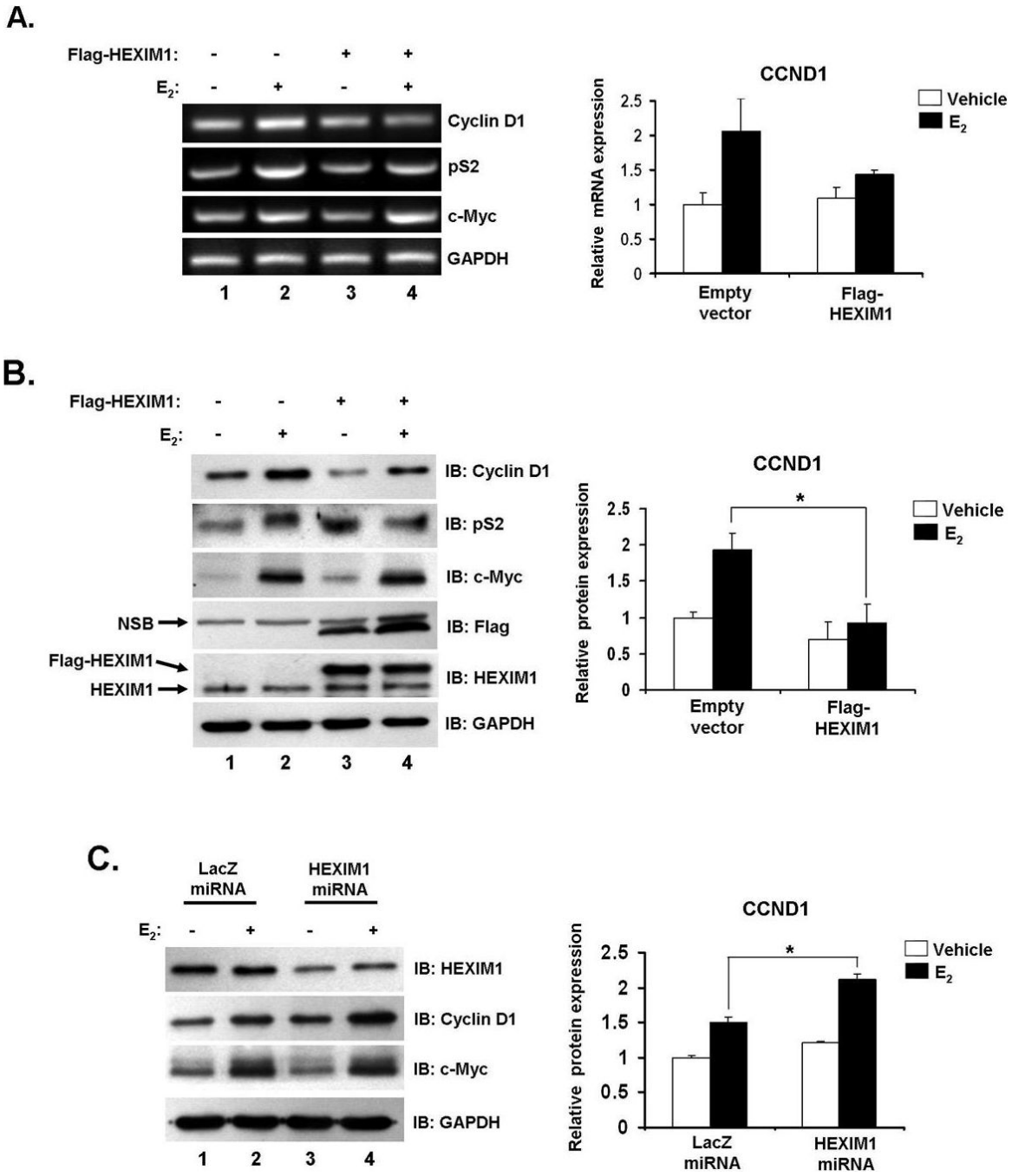

Figure 3. HEXIM1 regulates E_2-induced cyclin D1 expression in breast epithelial cells

A. MCF-7 cells were transfected with pCMV-Tag2B-HEXIM1 or empty vector, treated with 100 nM E_2 or ethanol vehicle for 3 hours. RNA was extracted and subjected to RT-PCR as described in "Materials and Methods". RT-PCR results quantitated for changes in cyclin D1 mRNA using GAPDH as a control. *Columns*, mean of four independent replicates; *bars*, SE; *, $P<0.05$

B. Representative Western blot using indicated antibodies for immunoblotting. Changes in cyclin D1 protein levels were quantitated and normalized to GAPDH. *Columns*, mean of three independent replicates; *bars*, SE; *, $P<0.05$

C. Representative Western blot indicating miRNA-mediated silencing of HEXIM1 in MCF-7 cells. E_2-induced changes in cyclin D1 between LacZ and HEXIM1 miRNA stable cells were quantitated and normalized to GAPDH. *Columns*, mean of three independent replicates; *bars*, SE; *, $P<0.01$

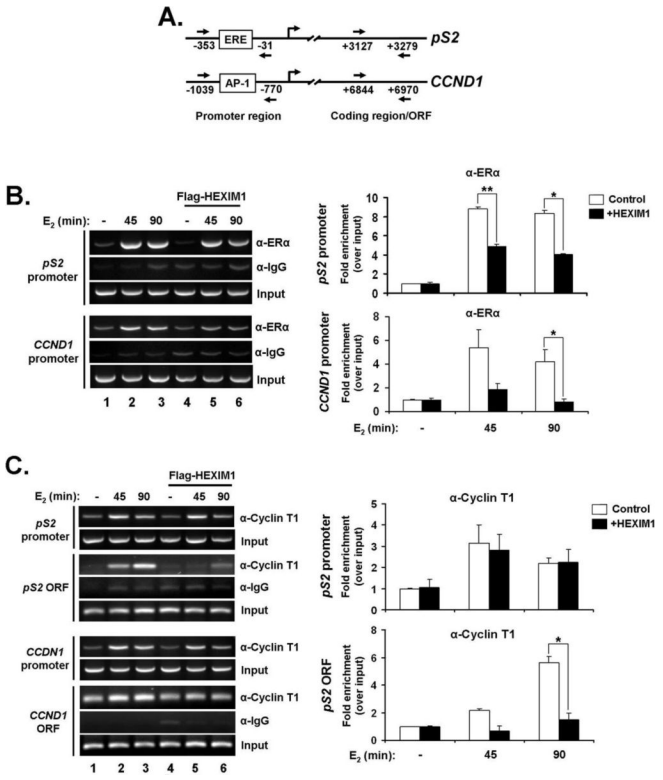

Figure 4. Effect of increased HEXIM1 expression on E_2-dependent recruitment of ERα and P-TEFb (cyclin T1) to ER-responsive genes

A. Primers used in ChIP assays are directed at regions indicated for *pS2* and *CCND1* genes.

B. Increased HEXIM1 expression leads to decrease in E_2/ERα recruitment to promoter regions of ER target genes, *pS2* and *CCND1*. Samples were prepared from MCF-7 cells transiently transfected with pCMV-Tag2B-HEXIM1 or empty vector, treated with ethanol vehicle or 100 nM E_2 for 45 and 90 minutes. Chromatin immunoprecipitation was done with antibodies against ERα and rabbit IgG (as an IP control). *Panels on the left*, DNA fragments were analyzed by PCR with primers specific for the promoter-proximal region of *pS2* and *CCND1* as indicated in (A). *Panels on the right*, Quantitations of ERα IP enrichment at *pS2* and *CCND1* promoters. *Columns*, mean of two independent replicates; *bars*, SE; *, $P<0.05$; **, $P<0.005$.

C. HEXIM1 inhibits E_2-dependent recruitment of cyclin T1 to coding regions of *pS2* and *CCND1*. MCF-7 cells were treated as described in (B). ChIP analysis of cyclin T1 recruitment to E_2-responsive region of *pS2* and *CCND1* promoter and coding regions was carried out using primers specific for the regions indicated in (A). *Panels on the left*, DNA fragments were analyzed by PCR with primers specific for the regions indicated. *Panels on the right*, Quantitations of cyclin T1 IP enrichment at *pS2* gene. *Columns*, mean of 2-3 independent replicates; *bars*, SE; *, $P<0.05$

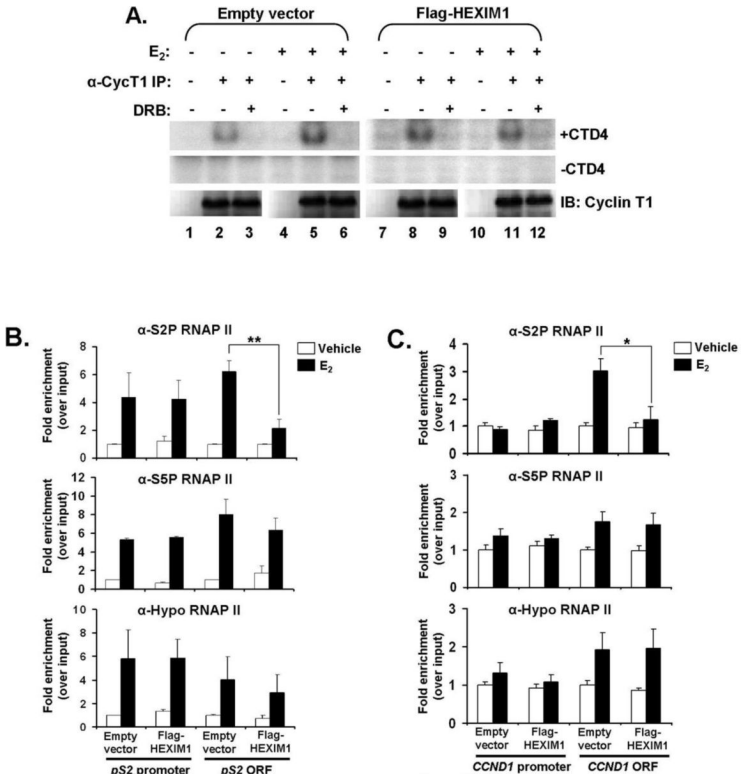

Figure 5. Increased HEXIM1 expression inhibits E_2-induced P-TEFb activity and recruitment of serine 2 (hyperphosphorylated) RNA polymerase II to the coding region of ER-responsive genes

A. Increased HEXIM1 expression decreased E_2-induced CTD4 peptide phosphorylation. MCF-7 cells were transiently transfected with pCMV-Tag2B-HEXIM1 or empty vector and treated with ethanol vehicle or 100 nM E_2 for 90 minutes. Cell lysates were subjected to immunoprecipitation with antibodies against cyclin T1 and rabbit IgG (as an IP control). The immunoprecipitates were divided into two halves with one half getting 2 μg of the CTD4 peptide added to the reaction (−/+ CTD4). Fifty μM DRB was also added to some immunoprecipitates as a kinase assay control. The kinase reactions were analyzed by SDS-PAGE using autoradiography. Equal volumes of kinase reactions were also analyzed by Western blot to check specificity of anti-cyclin T1 antibody in immunoprecipitation. Panels are representative of at least four independent experiments.

B. HEXIM1 inhibits E_2-dependent recruitment of S2P RNAP II to *pS2* ORF. MCF-7 cells were treated as described in Figure 4 and subjected to ChIP analysis with antibodies against serine 2 phosphorylated (S2P) RNAP II, serine 5 phosphorylated (S5P) RNAP II and the unphosphosphorylated form of RNAP II (8WG16). The regions of *pS2* amplified by PCR are as indicated. *Columns*, mean of 3-5 independent replicates; *bars*, SE; **, $P<0.005$

C. HEXIM1 inhibits E_2-dependent recruitment of S2P RNAP II to *CCND1* ORF. MCF-7 cells were treated as described. The regions of *CCND1* amplified by PCR are as indicated. *Columns*, mean of 3-4 independent replicates; *bars*, SE; *, $P<0.05$

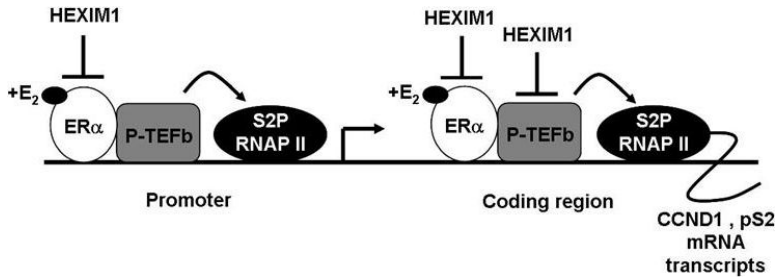

Figure 6.
Proposed model for HEXIM1 action on ERα and P-TEFb at ER-responsive genes, pS2 and CCND1, in mammary cells.