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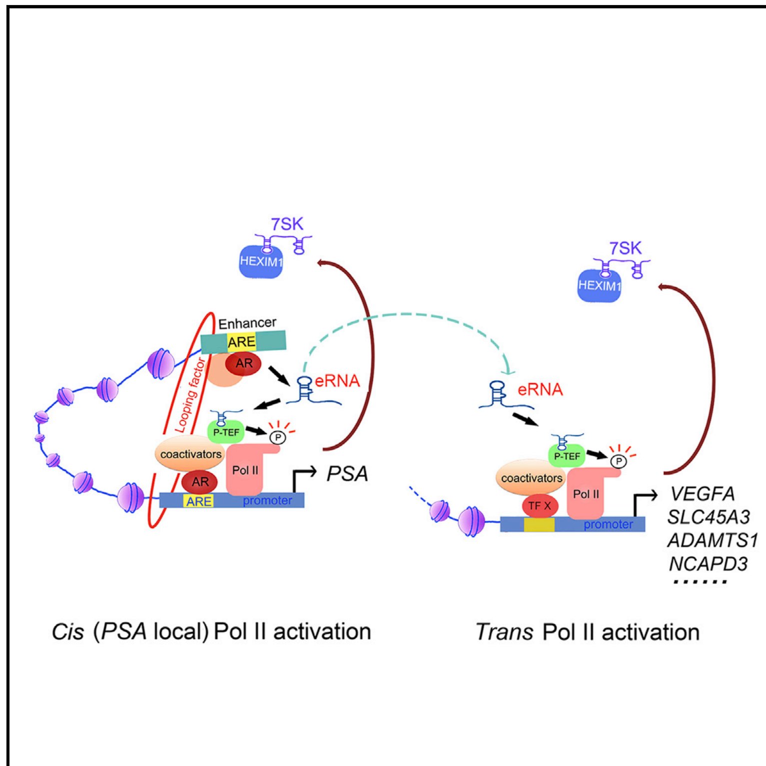
Activation of P-TEFb by Androgen Receptor-Regulated Enhancer RNAs in Castration-Resistant Prostate Cancer

Graphical Abstract



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In Brief

Zhao et al. show that a group of AR-regulated eRNAs, including the *PSA* eRNA, are upregulated in CRPC cells in culture as well as in patient specimens. The *PSA* eRNA binds to CYCLIN T1, activates P-TEFb, and increases Pol II-Ser2p and cell growth, and this effect is mediated through a TAR-L motif.

Highlights

- PSA eRNA is upregulated in CRPC cells in culture, PDXs, and patient tissues
- PSA eRNA binds to CYCLIN T1 and activates the P-TEFb complex
- PSA eRNA increases Pol II Ser2 phosphorylation
- A TAR-L motif in PSA eRNA is required for P-TEFb activation and CRPC growth

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Activation of P-TEFb by Androgen Receptor-Regulated Enhancer RNAs in Castration-Resistant Prostate Cancer

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SUMMARY

The androgen receptor (AR) is required for castration-resistant prostate cancer (CRPC) progression, but the function and disease relevance of AR-bound enhancers remain unclear. Here, we identify a group of AR-regulated enhancer RNAs (e.g., *PSA* eRNA) that are upregulated in CRPC cells, patient-derived xenografts (PDXs), and patient tissues. *PSA* eRNA binds to CYCLIN T1, activates P-TEFb, and promotes *cis* and *trans* target gene transcription by increasing serine-2 phosphorylation of RNA polymerase II (Pol II-Ser2p). We define an HIV-1 TAR RNA-like (TAR-L) motif in *PSA* eRNA that is required for CYCLIN T1 binding. Using TALEN-mediated gene editing we further demonstrate that this motif is essential for increased Pol II-Ser2p occupancy levels and CRPC cell growth. We have uncovered a P-TEFb activation mechanism and reveal altered eRNA expression that is related to abnormal AR function and may potentially be a therapeutic target in CRPC.

INTRODUCTION

Androgen deprivation therapy has long been the mainstay of treatment for advanced prostate cancer, but tumors inevitably become castration resistant (Debes and Tindall, 2004). Increasing evidence suggests that persistent androgen receptor (AR) signaling plays an essential role in development of hormone therapy resistance. A number of AR-centered mechanisms have been identified, including *AR* gene amplification, mutations in the AR ligand-binding domain, modulation of AR functions by upstream signaling pathways, and expression of *AR* splice variants (Feldman and Feldman, 2001; van der Steen et al., 2013). The discovery of castration-resistant prostate cancer (CRPC) dependence on the AR signaling has led to the development of second-generation endocrine therapeutics such as abiraterone and enzalutamide (de Bono et al., 2011; Scher et al., 2012). Enzalutamide acts to affect AR nuclear translocation and impairs AR transcriptional activation (Tran et al., 2009). Abiraterone is a cytochrome P450 17A1 (CYP17A1) inhibitor that blocks intratumoral androgen synthesis (Attard et al., 2008). Despite the increased benefit of the new regimens in overall survival, most patients develop resistance after initial responses (Scher et al., 2010); thus, a cure for CRPC remains elusive.

Developmental or tissue-specific gene expression is established primarily by transcription regulatory machineries at enhancers (Ong and Corces, 2011). Seminal works show that a class of non-coding RNAs, so-called enhancer RNAs (eRNAs), is widely transcribed from cell-lineage-specific enhancers (De Santa et al., 2010; Kim et al., 2010; Ørom et al., 2010; Wang et al., 2011). Further studies reveal that eRNAs are expressed in a large spectrum of cell types (Hah et al., 2015; Lam et al., 2013; Melo et al., 2013; Mousavi et al., 2013; Qian et al., 2014). In mouse cortical neurons, the abundance of eRNA at neuronal gene enhancers strongly correlates with the mRNA expression of nearby genes (Kim et al., 2010). Mechanistic studies show that, in a locus-specific manner, eRNAs act in *cis* to stabilize enhancer-promoter looping, establish chromatin accessibility, or facilitate release of the negative elongation factor (NELF) complex (Hsieh et al., 2014; Li et al., 2013; Mousavi et al., 2013; Schaukowitch et al., 2014). Estrogen-receptor (ER)- or

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AR-regulated eRNAs also act in *trans* to regulate gene expression in hormone-responsive cells, but the effects appear to be relatively infrequent in the cell types examined (Hsieh et al., 2014; Li et al., 2013).

A major step in regulation of gene transcription is the assembly of the preinitiation complex that brings RNA polymerase II (Pol II) to gene promoters. Intriguingly, Pol II is often paused around 20–60 nt downstream of the transcription start site (TSS). Pol II release is achieved primarily by the activation of the positive transcription elongation factor (P-TEFb), which promotes phosphorylation of at least three targets, including NELF, DRB-sensitivity-inducing factor (DSIF), and serine 2 in heptad repeats in the C-terminal domain (CTD) of the large subunit of Pol II (Pol II-Ser2) (Peterlin and Price, 2006; Zhou et al., 2012). P-TEFb is a heterodimer composed of cyclin-dependent kinase 9 (CDK9) and one of the C-type cyclins, including T1, T2a, and T2b, of which CYCLIN T1 is the most abundant partner (Peng et al., 1998). Approximately 50% of the total P-TEFb is present in an inactive form, due to the binding of the inhibitory ribonucleoprotein complex composed of 7SK small nuclear RNA (snRNA) and HEXIM1/2 (Nguyen et al., 2001; Yang et al., 2001). The *trans*-activating responsive (TAR) RNA at the 5′ end of the HIV-1 transcript and the 3′ hairpin of 7SK share a similar secondary structure (Egloff et al., 2006). TAR RNA interacts with the HIV-1 transcription activator Tat to form a protein complex that activates P-TEFb by competing away the inhibitory subunit 7SK-HEXIM1/2 (Egloff et al., 2006; Roy et al., 1990). BRD4 is another important activator of P-TEFb (Jang et al., 2005; Yang et al., 2005). BRD4 activates P-TEFb by recruiting JMJD6, which mediates the decapping/demethylation of 7SK and the dismissal of the 7SK/HEXIM1/2 inhibitory complex (Liu et al., 2013).

Significant progress has been made in elucidation of cellular functions of eRNAs in various tissue types. However, current knowledge about the mechanisms of action of eRNAs in gene transcription regulation and their roles in human diseases such as cancer is very limited. In the present study, we demonstrated that a group of AR-regulated enhancer RNAs (AR-eRNAs), such as *PSA* (or called *KLK3*) eRNA, are upregulated in CRPC cells in culture and patient specimens. We further showed that *PSA* eRNA binds to CYCLIN T1, activates P-TEFb, and increases Pol II-Ser2 phosphorylation (PolII-Ser2p). We found that this effect is mainly mediated through a TAR-L motif.

RESULTS

AR-eRNAs Are Upregulated in Human CRPC Cells and Patient Tissues

Given the pivotal role of AR function in CRPC progression, we sought to assess genome-wide AR activity by profiling AR-eRNA expression in CRPC cells. We used androgen-dependent LNCaP and its castration-resistant derivative C4-2 (Dehm and Tindall, 2006) as model systems. We performed strand-specific, ribosome-minus RNA sequencing (RNA-seq) in these cell lines. The hypersensitivity and large dynamic range of RNA-seq allowed us to identify and quantify the expression of both eRNAs and mRNAs. We performed, in parallel, AR chromatin immunoprecipitation and deep sequencing (ChIP-seq) to define the AR-binding sites in the genome. By assigning eRNAs to the nearest genes, we found that a total of 6,193 AR-eRNAs were expressed at AR-bound enhancers in both LNCaP and C4-2 cells (Figure 1A). A meta-analysis of published ChIP-seq data from LNCaP cells (Wang et al., 2011) indicates that these are authentic enhancers, as evident by the enrichment of AR coregulators (FOXA1, MED12, and P300), Pol II, H2AZ, and enhancer histone marks H3K4me1, H3K4me2, and H3K27ac (Figure 1A). Similar to a bimodal pattern of H3K4me2 at AR-bound enhancers (He et al., 2010), very low or no eRNA signals were detected in the center of the AR-binding sites but increased bilaterally, reminiscent of eRNA expression revealed by the global nuclear run-on sequencing (GRO-seq) data (Wang et al., 2011) (Figure 1A). In agreement with the previous report (Pekowska et al., 2011), H3K4me3 was also detectable at these transcriptionally active enhancers, but its level was much lower than that of the canonical enhancer histone marks (Figure 1A). The averaged RNA-seq signal intensities of 6,193 AR-eRNAs were globally higher in C4-2 cells compared to LNCaP cells when cultured under androgen deprivation conditions (Figure 1B). Unsupervised clustering analyses revealed that, among these AR-eRNAs, 1,865 (30%) were upregulated in C4-2 compared to LNCaP cells (Figures 1C and 1D), 923 (15%) were downregulated in C4-2, and 3,405 (55%) exhibited no statistically significant differences between these two cell lines. Upregulation of AR-eRNAs in C4-2 cells is exemplified by the well-studied AR target gene *PSA* (the third most highly expressed eRNA identified in C4-2 cells; see Table S1) and another AR target gene *ARHGEF26* (the most highly expressed eRNA; see Table S1) (Figure 1E). We conclude that expression of a large set of AR-eRNAs is aberrantly upregulated in C4-2 CRPC cells in culture.

To further investigate AR-eRNA expression in CRPC cells, we focused on the top five highly expressed cancer-relevant genes: *ARHGEF26*, *KLK15*, *HTR3A*, *TLE1*, and *SLC16A7* (Table S1). RNA-seq data revealed that eRNA peaks at these loci were intergenic (Figures 1E and S1A). Upregulation of these genes at the mRNA and protein levels in C4-2 CRPC cells and androgen regulation of their expression in androgen-sensitive LNCaP cells were confirmed by RT-qPCR and western blot (Figure 2A; Figure S1B). Substantial interactions between the promoters and the putative enhancers at these loci were validated by chromosome conformation capture (3C) assays in C4-2 and androgen-stimulated LNCaP cells (Figure 2B).

eRNA and mRNA expression at these loci were upregulated in CRPC patient-derived xenografts (PDXs) and patient samples compared to their hormone-naive counterparts (Figures 2C–2E and S1C). *PSA* eRNAs were also upregulated in CRPC patient samples (Figures 2D and 2E). Analysis of a previously reported dataset (Grasso et al., 2012) indicated that the expression of *HTR3A* and *TLE1* was particularly upregulated in metastatic CRPC patient samples compared to primary tumors (Figure S1D). We also analyzed mRNA expression of these five genes in two additional CRPC datasets in the public domain (Glinsky et al., 2005; Yu et al., 2004). We demonstrated that differential expression of these genes stratified prostate cancer patients into two subgroups, in which higher expression of these genes was associated with lower biochemical recurrence-free survival, and the discriminating power was consistent in these two independent cohorts of patients (Figure S1E). Thus, through

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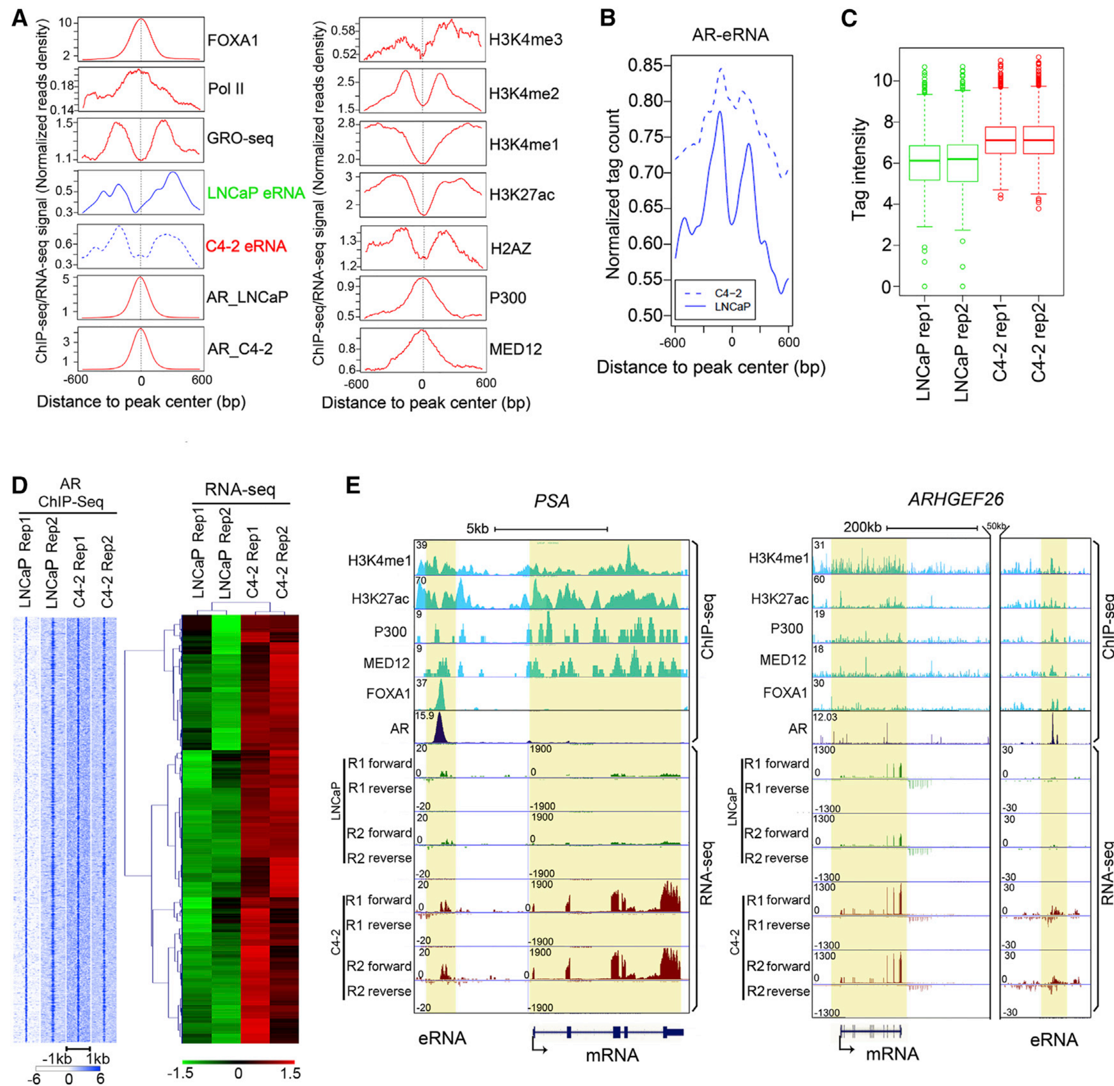

Figure 1. Upregulation of AR-eRNAs in CRPC Cells

(A) Aggregate plots showing the distribution of average tags (signal per million mapped reads) of AR ChIP-seq and RNA-seq around AR binding sites in LNCaP and C4-2 cells. Other signature profiles were obtained from LNCaP cells reported previously (Wang et al., 2011).

(B) Genome-wide comparison of AR-eRNA levels between LNCaP (solid line) and C4-2 (dashed line) cells.

(C) Box-and-whisker plot showing AR-eRNA signals upregulated in C4-2 at 1,865 AR-bound enhancers. $p < 2.2e-16$ (Wilcoxon test).

(D) Heatmap showing the unsupervised clustering of AR-eRNA signals upregulated in C4-2 cells at 1,865 AR-bound enhancers (right) and AR ChIP-seq signals within ±1 kb windows centered on AR binding sites (left).

(E) Screen shots from the UCSC genome browser showing signal profiles of eRNA expression in LNCaP and C4-2 cells and ChIP-seq in LNCaP cells. The eRNA and mRNA regions are highlighted in yellow. Left: *PSA*. Right: *ARHGEF26*.

eRNA profiling analyses, we identified a subset of AR-regulated genes whose expression is associated with CRPC progression in patients.

PSA eRNA Regulates Pol II-Ser2p in the *cis* Locus

We found that the AR was required for AR-eRNA expression in C4-2 cells (Figure S1F). However, upregulation of AR-eRNAs in C4-2 cells was unlikely caused by increased expression of AR protein, because little or no difference in AR protein level was detected in C4-2 and LNCaP cells, regardless of androgen treatment (Figure S1G), which is consistent with the previous report (Dehm and Tindall, 2006). No significant correlation was observed between the expression of AR-eRNAs (or corresponding mRNAs) and AR binding at the 1,865 enhancers where AR-eRNAs were upregulated in C4-2 cells (Figure S1H). ChIP assays demonstrated that active histone modifications such as H3K4me1 and H3K27ac were upregulated, but the repressive histone modification H3K9me2 was downregulated in the AR-eRNA-expressing enhancers examined in C4-2 compared to LNCaP cells (Figure S1I). Thus, it appears that the active histone

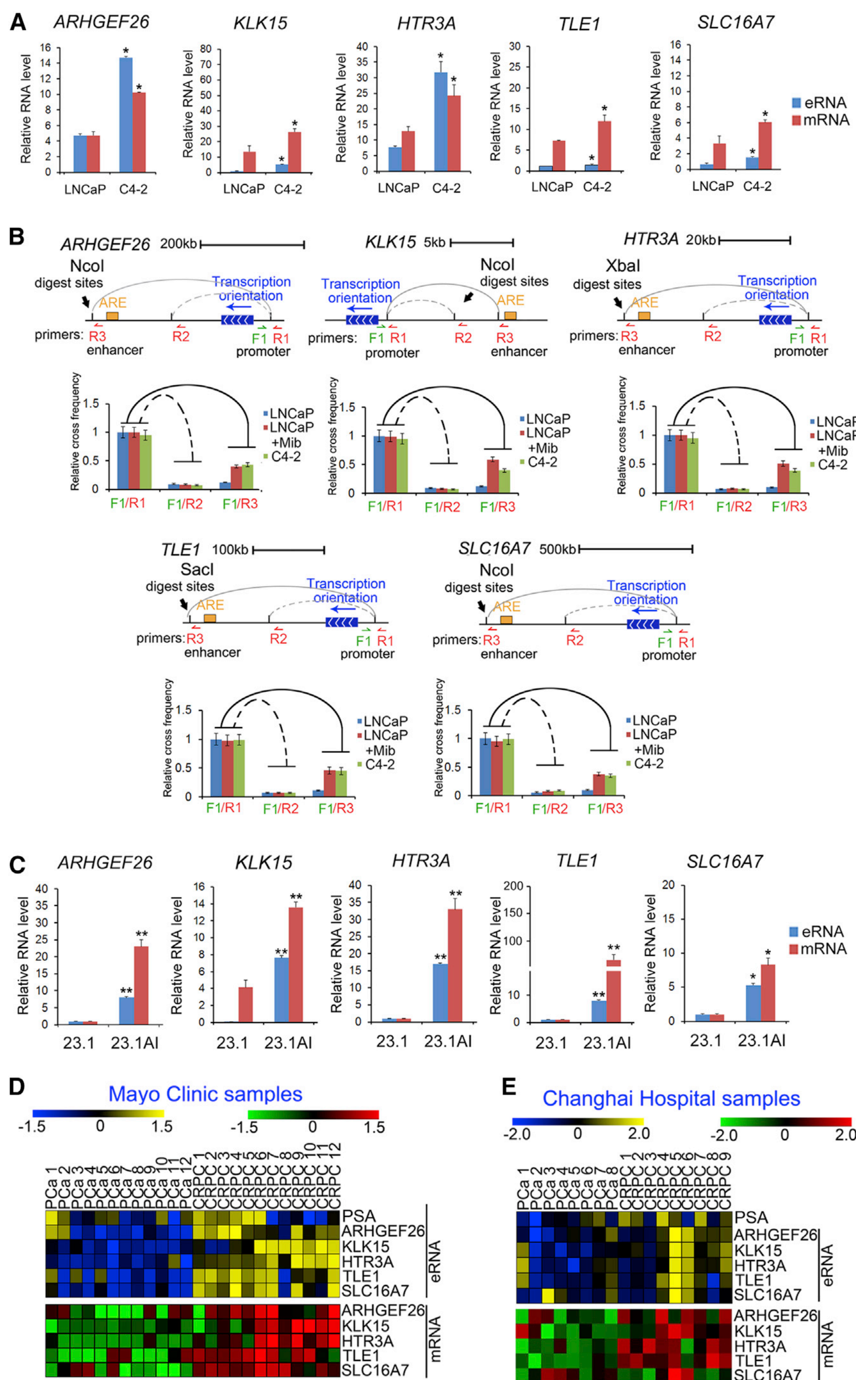

Figure 2. Identification of Five New AR Target Genes Whose Expression Is Upregulated in CRPC Tissues

(A) eRNA expression and mRNA expression of five new AR target genes (*ARHGEF26*, *KLK15*, *HTR3A*, *TLE1*, and *SLC16A7*) were measured by RT-qPCR. Data indicate means ± SD (n = 3). *p < 0.05.

(B) Evaluation of the enhancer-promoter interaction at novel AR target gene loci by 3C assays. F, forward primer; R, reverse primer; Mib, Mibolerone.

(C) eRNA expression and mRNA expression of five new AR target genes were measured by RT-qPCR in androgen-dependent (23.1) and castration-resistant (23.1AI) PDXs grown in mice. Data indicate means ± SD (n = 3).*p < 0.05; **p < 0.01.

(D and E) Heatmap of PSA eRNA and eRNA/mRNA expression of five new AR target genes in human primary prostate cancer (PCa) and CRPC tissues obtained from Mayo Clinic (D) and Shanghai Changhai Hospital (E) (PSA eRNA expression in CRPC versus PCa, p = 0.000264 and p = 0.027184, respectively).

modifications, but not the AR protein level, are responsible for the increased expression of AR-eRNAs in C4-2 CRPC cells.

Similar to the previous findings in cortical neurons (Kim et al., 2010), our RNA-seq data revealed a global correlation between eRNA and mRNA expression in both LNCaP (r = 0.59) and C4-2 (r = 0.56) cells (Figure S2A). Therefore, we sought to determine the functional importance of AR-eRNAs in the regulation of AR target gene mRNA expression in CRPC cells. Knockdown of *PSA* eRNA by sequence-specific small interfering RNAs (siRNAs) significantly decreased *PSA* mRNA expression in C4-2 cells (Figure S2B). To avoid the potential off-target effect of siRNAs, highly optimized generation-2.5 antisense oligonucleotides (ASOs) (Burel et al., 2013), which induce RNase-H-dependent degradation of the complementary target RNA without involving the cellular RNAi machinery, were used as an independent approach to knock down *PSA* eRNA. The levels of *PSA* eRNAs, unlike the other AR-eRNAs examined, were effectively reduced by two independent ASOs of *PSA* eRNAs (Figure 3A; Figure S2C). Similar to siRNAs, treatment of C4-2 cells with ASOs also significantly reduced *PSA* mRNA expression (Figure 3A). The consistent results obtained from two independent knockdown methods suggest a *cis* function of *PSA* eRNA in regulating *PSA* mRNA expression in CRPC cells.

Pol II-Ser2p is required for productive transcription elongation and gene expression (Zhou et al., 2012). Therefore, we examined whether *PSA* eRNA expression affects Pol II-Ser2p at the *PSA* locus. Knockdown of *PSA* eRNA by siRNAs decreased the Pol II-Ser2p level in the *PSA* promoter in both LNCaP and C4-2 cells (Figure S2D). Similarly, knockdown of *PSA* eRNAs by ASOs resulted in a decrease in Pol II-Ser2p at the *PSA* promoter in C4-2 cells, which was accompanied by an increase in total Pol II, although no overt change in unphosphorylated Pol II was detected (Figures 3B and S2E). While Pol II promoter proximal pause release and Pol II-Ser2p are achieved mainly through

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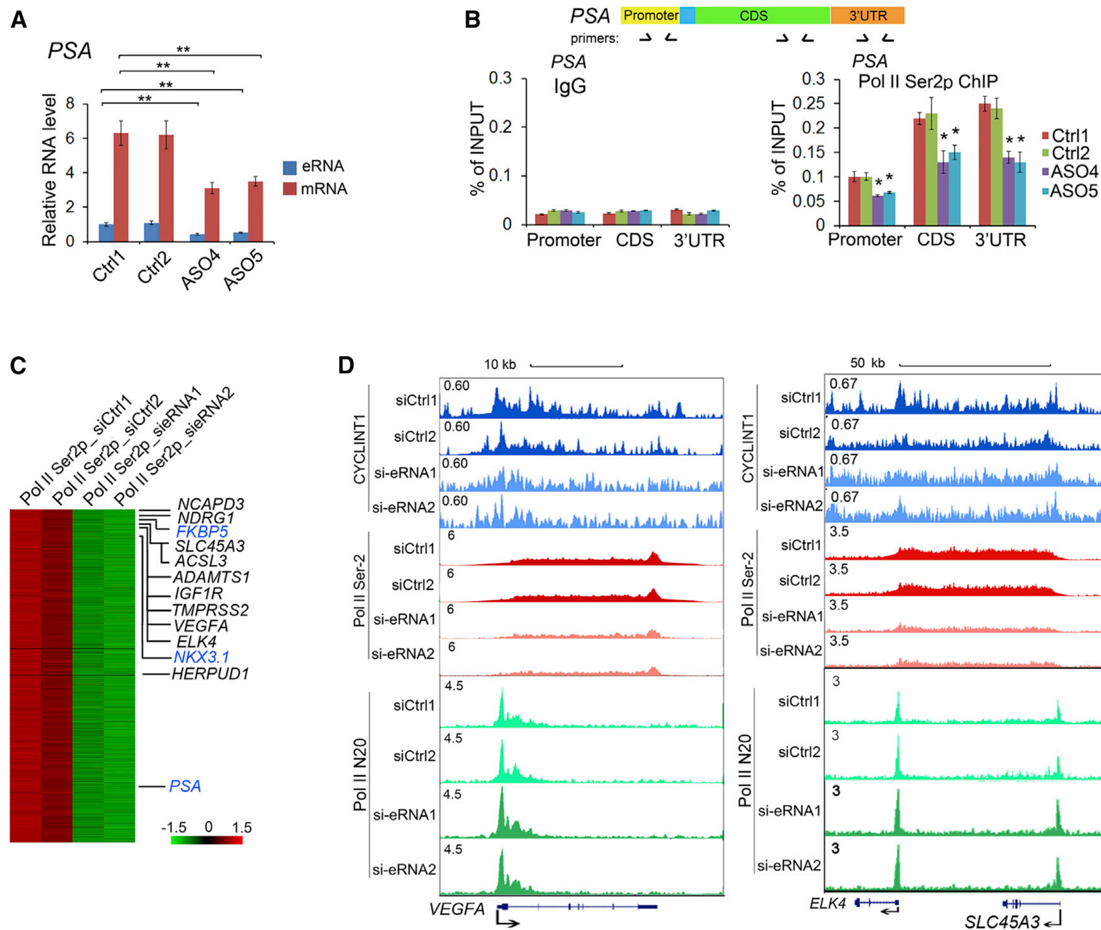

Figure 3. *PSA* eRNA Regulates Pol II-Ser2p at Both *cis* and *trans* Targets

(A) Effectiveness of *PSA* eRNA knockdown mediated by sequence-specific ASOs and its effect on *PSA* mRNA expression. Data indicate means ± SD (n = 3). $^{**}p < 0.01$. Ctrl, control.

(B) ChIP-qPCR analysis of Pol II-Ser2p at the *PSA* gene locus in C4-2 cells. Immunoglobulin G (IgG) was used as a control. ChIP DNA was analyzed by real-time PCR. Data indicate means ± SD (n = 3). $^{*}p < 0.05$, compared to control 1.

(C) Heatmap of 674 gene loci showing Pol II-Ser2p signals were downregulated in C4-2 cells transfected with *PSA* eRNA siRNAs.

(D) Screen shots from the UCSC (University of California, Santa Cruz) Genome Browser showing ChIP-seq signal profiles of Pol II-Ser2p, total Pol II, and CYCLIN T1 binding in C4-2 cells transfected with control or *PSA* eRNA siRNAs. Left: *VEGFA*. Right: *SLC45A3*.

the action of P-TEFb composed of CYCLIN T and CDK9, we found that *PSA* eRNA knockdown had little or no effect on CYCLIN T1 binding in the *PSA* promoter (Figure S2F). Moreover, AR ChIP-seq analyses showed that *PSA* eRNA knockdown in C4-2 cells slightly decreased AR binding at the *PSA* promoter, but the decrease was not statistically significant (Figure S2G). This result was further confirmed by AR ChIP-qPCR (Figure S2G). Reduction in *PSA* eRNA also decreased Pol II-Ser2p occupancy levels at genomic areas corresponding to the coding sequence (CDS) and 3′ UTR (Figure 3B). These data suggest that *PSA* eRNA plays a critical *cis* role in regulating Pol II-Ser2p levels at the *PSA* gene locus.

PSA eRNA Regulates Pol II-Ser2p at *trans* Target Loci

Most ER eRNAs examined are expressed at very low levels (5–15 copies per cell) in breast cancer cells, and the *trans* effects of ER eRNAs are relatively infrequent (Li et al., 2013). Given that the *trans* effects of *PSA* eRNA were observed in androgen-sensitive LNCaP cells (Hsieh et al., 2014), we measured the copy numbers of *PSA* eRNA in LNCaP and C4-2 cells. *PSA* eRNA levels were low (52 ± 11 copies per nucleus) in LNCaP cells but were >15-fold higher (831 ± 86 copies per nucleus) in C4-2 cells (Figure S2H). These results prompted us to determine the global *trans* effect of *PSA* eRNA knockdown on Pol II-Ser2p levels. We performed Pol II-Ser2p, as well as total and unphosphorylated Pol II ChIP-seq, in mock and *PSA* eRNA siRNA-treated C4-2 cells. Given that P-TEFb (CYCLIN T1 and CDK9) is the major factor responsible for Pol II-Ser2p, we also performed CYCLIN T1 ChIP-seq. *PSA* eRNA knockdown significantly decreased Pol II-Ser2p levels at the loci of a subset of genes (674 targets, false discovery rate [FDR] < 0.001), which include the *cis* target *PSA* and the known (*FKBP5* and *NKX3.1*) (Hsieh et al., 2014) and new *trans* targets, with functions in regulation of cell cycle, growth, survival, migration, and invasion, such as

VEGFA, *NCAPD3* (Liu et al., 2010), *ADAMTS1* (Carver et al., 2009), and *IGF1R* (Figures 3C, 3D, and S2I; Table S2). *PSA* eRNA knockdown had little or no effect on occupancy of unphosphorylated Pol II and AR at the 674 targets (Figures S2J and S2K). While Pol II-Ser2p was apparently reduced by *PSA* eRNA knockdown at these loci, overall changes in total Pol II (including increase in the promoter and decreased occupancy in the gene body, as measured by traveling ratio) were relatively small (Figures S2K–S2M), making the explanation likely to be the changes in both transcription and processing. Indeed, consistent with the previous finding in *Drosophila* that decreased Pol II-Ser2p decreases mRNA levels by affecting mRNA 3′ processing (Ni et al., 2004), we found that the ratio of cleaved to uncleaved RNA in the control knockdown cells was much greater than that in *PSA* eRNA knockdown cells at three out of five loci examined (Figure S2N). These data suggest that the effect of *PSA* eRNA knockdown on gene expression can be attributed to a combination of reduced transcription and defective 3′ processing.

Little or no change in CYCLIN T1 binding in the majority (498 of 674, approximately 74%) of these loci was detected consistently among the replicates (Table S3). Notably, 586 out of 674 loci (87%) had AR binding within 50 kb around the TSS (Table S2), and among the top 25 hits were known AR-regulated genes (e.g., *NDRG1*, *FKBP5*, *SLC45A3*, and *TMPRSS2*) (Figure 3C; Table S2), suggesting the importance of *PSA* eRNA in regulating AR signaling. ChIP-qPCR and RT-qPCR assays further confirmed that knockdown of *PSA* eRNA by two independent ASOs invariably decreased Pol II-Ser2p levels at the promoters of the *trans* targets examined as well as mRNA expression of these genes in C4-2, but not in the non-prostatic cell lines examined (Figures S3A–S3C). Chromatin isolation by RNA purification (ChIRP) assays showed that *PSA* eRNA was present in the promoters of these genes (Figure S3D). The effects of *PSA* eRNA on total, unphosphorylated, and Ser5-phosphorylated Pol II, CYCLIN T1, and AR binding at the promoters of these *trans* target loci were validated by ChIP-qPCR (Figure S3E). *PSA* eRNA knockdown mitigated enhancer-promoter looping in *cis* (*PSA*) but not in *trans* loci (*TMPRSS2* and *NKX3.1*) (Figure S3F). These data indicate that *PSA* eRNA regulates Pol II-Ser2p levels at a number of *trans* target loci.

PSA eRNA Binds to CYCLIN T1 of the P-TEFb Complex

In agreement with the high expression of *PSA* eRNA in C4-2 cells (Figure 1E) and the role of *PSA* eRNA in the regulation of Pol II-Ser2p (Figures 3B–3D), CDK9-mediated Pol II-Ser2p was much higher in C4-2 than that in LNCaP cells (Figure S4A). Besides P-TEFb, other factors such as CDK12 and the phosphatase FCP1 are also involved in the regulation of Pol II-Ser2p (Cho et al., 2001; Davidson et al., 2014; Zhou et al., 2012). While dissociation of NELF from the paused Pol II cannot affect Pol II-Ser2p, it plays an essential role in promoting transcription elongation (Yamaguchi et al., 1999). Given that eRNA induces transcription elongation by binding to and causing the release of the E subunit of NELF (NELF-E) from the paused Pol II (Schaukowitch et al., 2014), we examined whether *PSA* eRNA binds to these factors. A biotin-labeled RNA pulldown assay demonstrated that only CDK9 and CYCLIN T1 were associated with *PSA* eRNA, and these results were confirmed by reciprocal RNA immunoprecipitation (RIP) assays (Figures 4A and 4B). In vitro kinase assays demonstrated that *PSA* eRNA enhanced P-TEFb complex-mediated phosphorylation of Pol II CTD (Figure 4C). Accordingly, concomitant *PSA* eRNA and CDK9 knockdown failed to further decrease expression of the *cis* and *trans* targets of *PSA* eRNA, compared with CDK9 knockdown alone (Figure S4B), suggesting that the function of *PSA* eRNA is mainly mediated through P-TEFb. Consistent with the GRO-seq results (Wang et al., 2011), our RNA-seq data showed that high-level eRNA signals were mainly detected in a 350-nt peak region (Figures 1E and S4C). In vitro RNA binding assays demonstrated that GST (glutathione S-transferase)-CYCLIN T1, but not GST-CDK9 or GST alone, preferentially bound to RNAs that were in vitro transcribed from the eRNA peak region, in comparison to those synthesized from a control region (Figures S4C and S4D). A similar specific interaction between the *PSA* eRNA peak region and endogenous CYCLIN T1 was detected in C4-2 cells using sonication RIP assay (Figure 4D). ChIRP and ChIP assays demonstrated that *PSA* eRNA and CYCLIN T1 bound to the same region of the *PSA* promoter (Figure 4E).

Like other CDKs, threonine 186 phosphorylation (T186p) in the T-loop is required for CDK9 activation (Li et al., 2005), and CDK7 is responsible for this phosphorylation (Larochelle et al., 2012). While CDK7 knockdown decreased T186p in both LNCaP and C4-2 cells (Figure S4E), little or no difference in the levels of CDK9 T186p was detected in these two cell lines (Figure S4F). Knockdown of *PSA* eRNA had no effect on T186p in C4-2 cells (Figure S4G). Notably, previous findings show that, while T186p is essential for CDK9 activation, T186-phosphorylated CDK9 can be functionally inactive, due to its binding by 7SK and HEXIM1/2, the inhibitory subunit of P-TEFb (Zhou et al., 2012). Thus, *PSA* eRNA may regulate Pol II Ser2p and the P-TEFb activity through mechanisms dependent on CYCLIN T1 binding but independent of CDK9 T-loop phosphorylation.

Identification of a TAR-L Motif in *PSA* eRNA that Promotes P-TEFb Activation

We demonstrated that deletion of a 50-bp region (−3,904 to −3,854 relative to TSS) within the *PSA* eRNA peak region largely diminished *PSA* eRNA binding to the recombinant CYCLIN T1 protein in vitro and the CYCLIN T1-CDK9 protein complex in cells (Figure 5A; Figure S5A). Deletion of the same region also significantly abolished the transcriptional activity of the *PSA* reporter gene (Figure 5B). It is worth noting that, while deletion of the −3,904/−3,854 region abrogated *PSA* eRNA binding with the CYCLIN T1/CDK9 complex in vitro, it did not completely abolish *PSA* reporter gene activity in cells (Figures 5A and 5B). A plausible reason is that the intact endogenous *PSA* eRNA may act in *trans* to activate the reporter gene in the presence of endogenous CYCLIN T1/CDK9 complex. This concept was further supported by the finding that the transcriptional activity of a promoter-alone reporter gene, which lacks any potential *cis* elements from the enhancer region, was also diminished by *PSA* eRNA knockdown (Figure S5B).

Within the CYCLIN T1-binding region (−3,904 to approximately −3,854) of *PSA* eRNA, we identified a TAR RNA-like

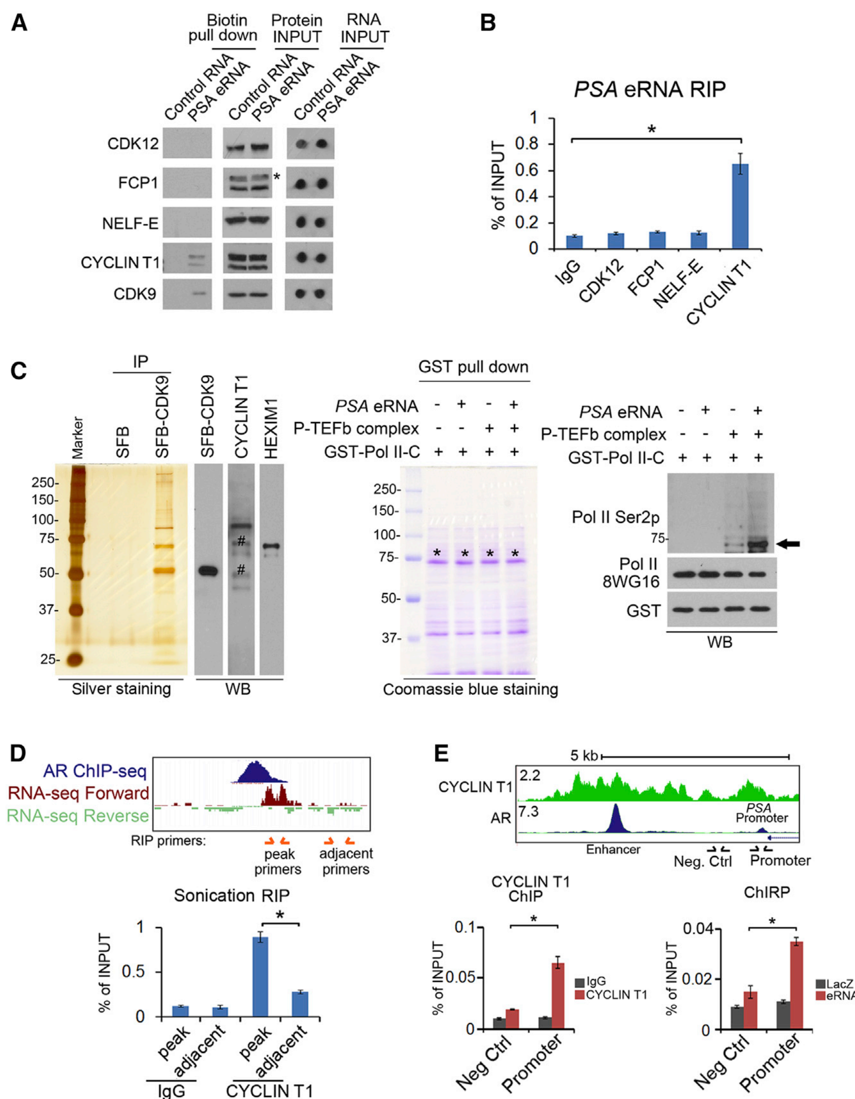

Figure 4. *PSA* eRNA Binds to P-TEFb

(A) C4-2 cell lysate was incubated with in-vitro-transcribed, biotin-labeled RNAs followed by western blots. Input RNA was analyzed by dot-blot hybridization. Asterisk indicates non-specific band.

(B) C4-2 cell lysate was incubated with IgG, CDK12, FCP1, NELF-E, or CYCLIN T1 antibodies for RIP, and RIP RNAs were analyzed by RT-qPCR. Data indicate means ± SD (n = 3). $^{*}p < 0.05$.

(C) Left: silver staining of SFB-CDK9 immunoprecipitated from 293T cells and western blot (WB). Pound signs indicate nonspecific bands. Middle: Coomassie blue staining of GST-Pol II-CTD (asterisks) used for kinase assay. Right: in vitro CDK9 kinase assay followed by western blot. IP, immunoprecipitate.

(D) Top: schematic diagram of the *PSA* eRNA peak region and primers for RIP assay. Bottom: sonicated C4-2 cell lysate was subjected to RIP with IgG or CYCLIN T1 antibodies and RT-qPCR. $^{*}p < 0.05$.

(E) ChIP assay using IgG or CYCLIN T1 antibodies or ChIRP assay using biotin-labeled LacZ or *PSA* eRNA-specific DNA probes and streptavidin beads in C4-2 cells. Neg Ctrl, negative control.

For (D) and (E), real-time PCR data indicate means ± SD (n = 3). $^{*}p < 0.05$.

(TAR-L) motif that shares a similar secondary structure with TAR RNA and the 3′ end of 7SK snRNA (Figures 5C, S5C, and S5D). Deletion of the TAR-L motif largely diminished *PSA* eRNA binding with CYCLIN T1 in vitro (Figure 5D), suggesting that TAR-L is important for CYCLIN T1 binding. We further showed that the in vitro and in vivo interactions of these molecules were both largely diminished by the addition or expression of 7SK, respectively (Figures 5E and S4D). No association of *PSA* eRNA with HEXIM1 was detected by RIP assay in C4-2 cells (Figure 5E). ChIP assays demonstrated that knockdown of *PSA* eRNA by ASOs increased HEXIM1 binding in the promoters of both *cis* and *trans* targets of *PSA* eRNA (Figures S5E and S5F). These data suggest that *PSA* eRNA promotes P-TEFb activation by functioning as a competitor of 7SK.

To further characterize the function of the TAR-L motif, we generated a unique *PSA* reporter gene construct, PSA-5′Insert-Luc, by inserting a 15-bp non-specific sequence in the region 5′ to the enhancer androgen-responsive element (ARE). This insertion did not affect the transcriptional activity of the reporter but makes the reporter construct suitable for ChIP assessment of AR binding (Figure S5G). Deletion of TAR-L impaired the transcriptional activity of this reporter gene, and the effect was reversed by replacing TAR-L with TAR RNA (nt 20–42) or the 3′ end (nt 302–324) of 7SK (Figure 5F). However, reversing the orientation of the TAR-L motif inhibited the reporter gene activity (Figure 5F). Similarly, replacement of TAR-L with TAR or the 3′ end of 7SK in a reversed orientation failed to restore the reporter gene activity (Figure 5G), which is consistent with the previous report that the orientation of 7SK and TAR is functionally important (Dingwall et al., 1990). These data argue that the effect of TAR-L deletion was unlikely caused by loss of any potential *cis*-regulatory element within this region. These genetic manipulations did not affect AR binding to the *PSA* enhancer and promoter of the reporter gene (Figure S5H). Disruption of the hairpin structure by introducing mutations in the complementary base pairs in the TAR-L motif impaired *PSA* promoter transcriptional activity in both C4-2 and LNCaP cells (Figure S5I). Putative TAR-L sequences were also identified in other highly expressed AR-eRNAs (Figure S5J). Similar to the *PSA* eRNA, the TAR-L motif in *TMPRSS2* and *SLC16A7* eRNAs was important for CYCLIN T1 binding in vitro (Figure S5K). They also bound to CYCLIN T1 in cultured cells, and their binding was largely diminished by 7SK expression (Figure S5L). Thus, we have identified a TAR-L module in AR-eRNAs that is critical for eRNA

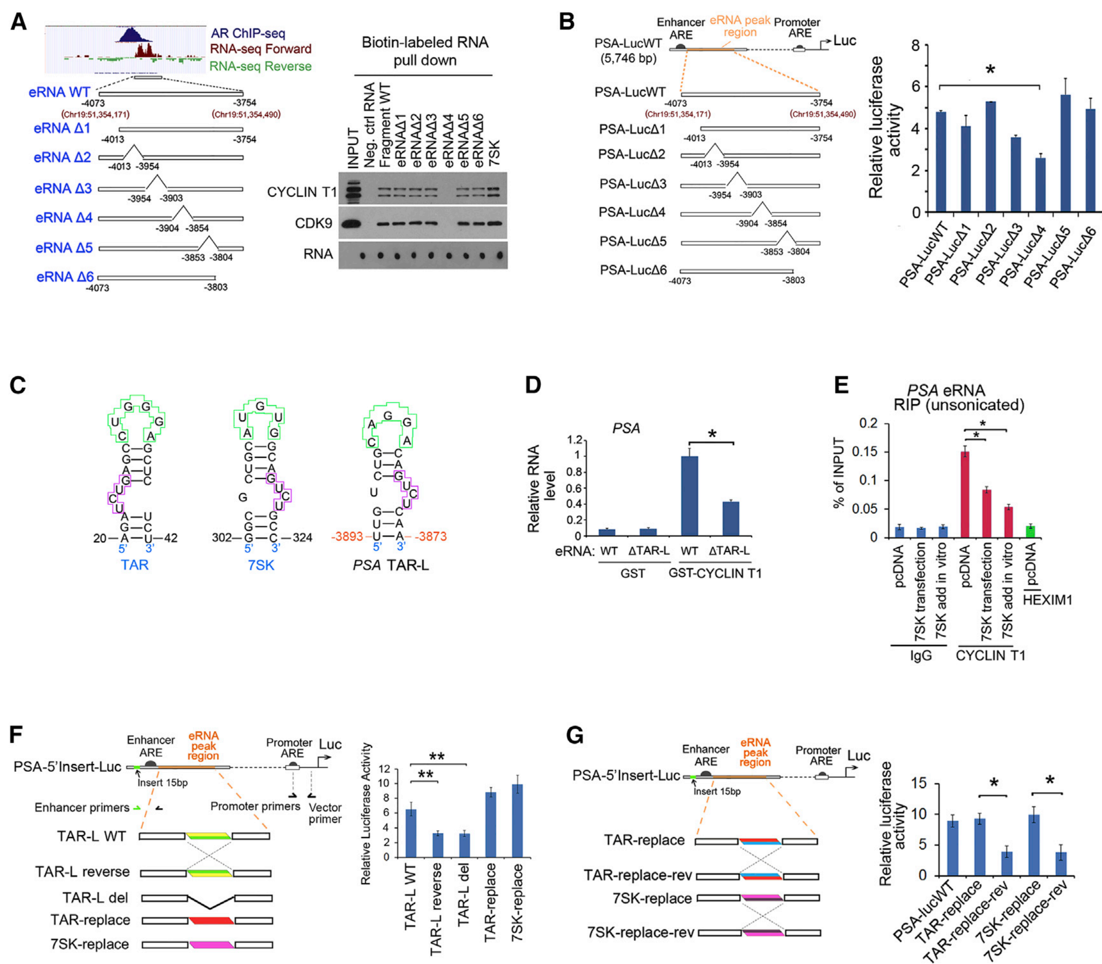

Figure 5. Identification of a TAR-L Motif in *PSA* eRNA Important for P-TEFb Binding and Gene Transactivation

(A) Left: the broken lines represent the deletion regions in *PSA* eRNA. Right: in-vitro-transcribed wild-type and mutated *PSA* eRNAs were incubated with C4-2 cell lysates. eRNA-bound proteins and RNA inputs were subject to SDS-PAGE and dot-blot hybridization, respectively. Neg. ctrl, negative control.

(B) Measurement of the luciferase activity of PSA-Luc reporter constructs (containing an eRNA region that was either wild-type or mutated) in C4-2 cells. Data indicate means ± SD (n = 3). *$p < 0.05$.

(C) Structural comparison of the HIV-1 TAR RNA, the 3′ end of 7SK snRNA and the TAR-L motif in *PSA* eRNA.

(D) Examination of CYCLIN T1 binding to wild-type and mutated *PSA* eRNA. *PSA* eRNA pulled down by GST or GST-CYCLIN T1 was detected by RT-qPCR. Data indicate means ± SD (n = 3). *$p < 0.05$.

(E) C4-2 cells were transfected with pcDNA3.1 or pcDNA3.1-7SK plasmids or lysate of untransfected cells was added with in vitro transcribed 7SK prior to RIP assay. Data indicate means ± SD (n = 3). *$p < 0.05$.

(F and G) Left: schematic diagram of the *PSA*-5′ Insert-Luc report construct containing an eRNA region. The fragment in orange represents the *PSA* eRNA peak region. Right: luciferase measurement in C4-2 cells. Data indicate means ± SD (n = 3). *$p < 0.05$; **$p < 0.01$. Del, deletion; Rev, reverse.

binding to CYCLIN T1, P-TEFb activation, and eRNA-mediated AR target gene transcription.

Importance of the Endogenous TAR-L Element in P-TEFb Activation in Cells

To further our understanding of the function of the TAR-L motif in *PSA* eRNA, we used TALEN-based DNA editing (Bedell et al., 2012) to specifically delete the TAR-L motif or an adjacent region (negative control) in the genome of C4-2 cells (Figure S6A). Similar to LNCaP, C4-2 cells are tetraploid (4n) with four copies of chromosome 19 where the *PSA* gene is located (Murillo et al., 2006). We acquired three independent TAR-L partial (50%) deletion subclones in which this motif was completely removed and replaced by non-specific sequences (Figures S6B–S6D and S6G). We also obtained two control subclones where the target sequence was partially (50% or 75%) deleted, and both deletions had no significant effect on *PSA* eRNA and mRNA expression in C4-2 cells (Figures S6E–S6H). While TAR-L deletion had little or no effect on *PSA* eRNA expression (Figure 6A), it significantly downregulated *PSA* mRNA expression, decreased Pol II-Ser2p levels at the *PSA* locus, and increased total Pol II and HEXIM1 binding to the *PSA* promoter, but it did not affect AR and CYCLIN T1 binding, unphosphorylated Pol II levels, and enhancer/promoter looping

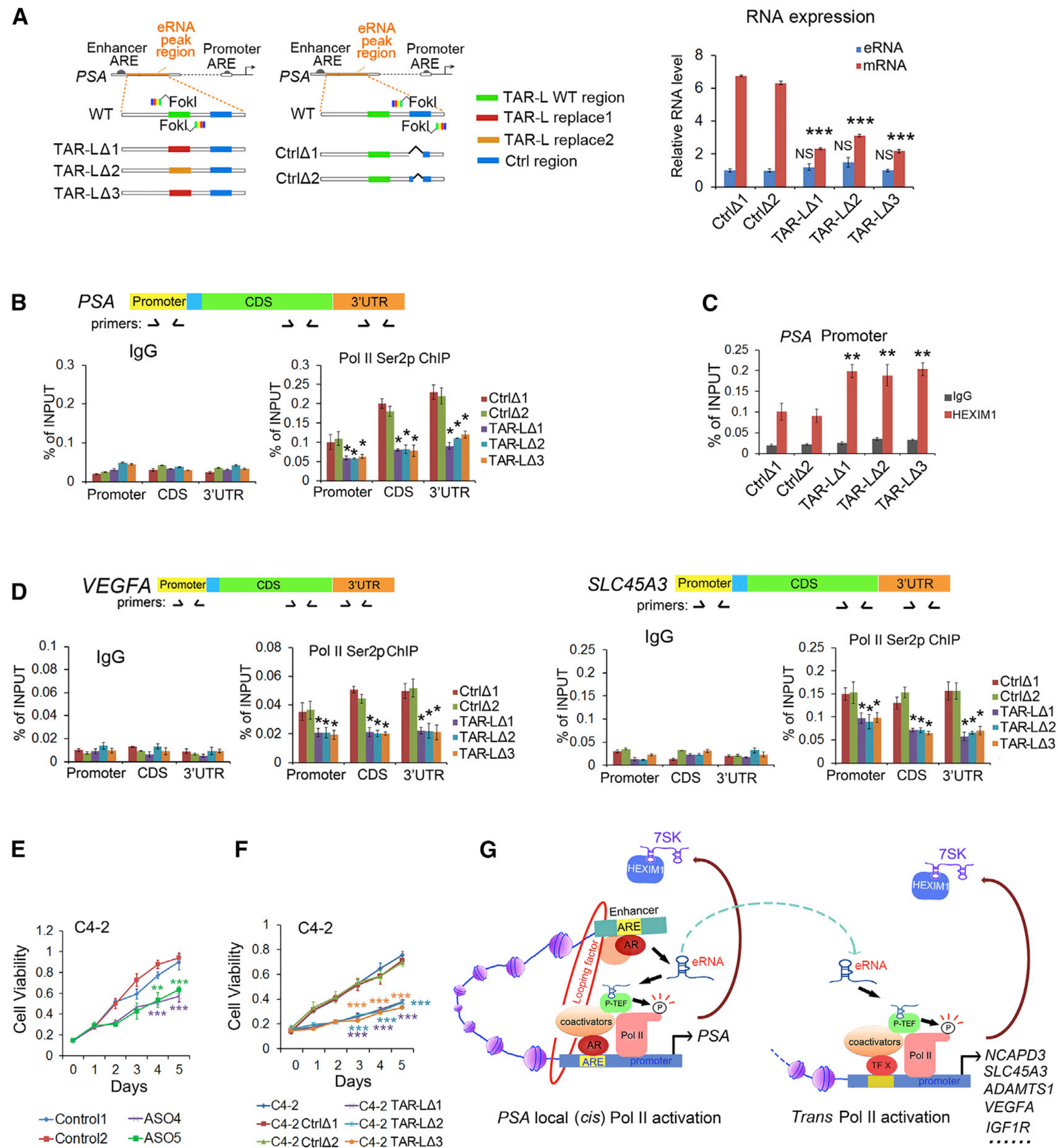

Figure 6. The TAR-L Motif in the Endogenous *PSA* eRNA Is Important for Its Target Gene Expression, Pol II-Ser2p Levels, and CRPC Cell Growth

(A) Left: schematic diagram showing C4-2 cell clones expressing *PSA* eRNA in which the TAR-L motif or a control (Ctrl) region was deleted by TALENs. WT, wild-type. Right: expression of *PSA* eRNA and mRNA in C4-2 TAR-L deletion stable cells measured by RT-qPCR. Data indicate means ± SD (n = 3). ***p < 0.001, compared to control Δ1. NS, not significant.

(B) ChIP-qPCR analysis of Pol II Ser2p at the *PSA* gene locus in C4-2 TALEN-modified cells. Data indicate means ± SD (n = 3). *p < 0.05, compared to control Δ1.

(C) ChIP-qPCR analysis of HEXIM1 binding at the *PSA* promoter. ChIP DNA was analyzed by real-time PCR. Data indicate means ± SD (n = 3). **p < 0.01, compared to control Δ1.

(D) ChIP-qPCR analysis of Pol II-Ser2p at the *trans* target loci *VEGFA* and *SLC45A3* in C4-2 TALEN-modified cells. Data indicate means ± SD (n = 3). *p < 0.01, compared to control Δ1.

(E and F) Viability of mock or ASO-transfected, or control or TALEN-modified, C4-2 cells was measured by MTS assay. Data indicate means ± SD (n = 6). **p < 0.01; ***p < 0.001, compared to control 1.

(G) A hypothetic model of *PSA* eRNA-mediated *cis*- and *trans*-activation of the P-TEFb complex and gene transcription.

(Figures 6A–6C and S6I–S6L). Similar results were obtained for the *PSA* eRNA *trans* targets examined (Figures 6D, S6L, and S7A–S7F). Moreover, *PSA* eRNA expression was correlated with mRNA expression of these *trans* target genes in CRPC patient samples (Figure S7G). We conclude that the TAR-L motif is required for *PSA* eRNA-mediated regulation of Pol II-Ser2p

levels at both *cis* and *trans* target loci and their mRNA expression in CRPC cells.

We demonstrated that knockdown of *PSA* eRNA (not *PSA* mRNA) by ASOs or siRNA not only specifically decreased the viability of C4-2 CRPC cells but also enhanced the inhibitory effect of the antiandrogen bicalutamide rather than enzalutamide (Figures 6E, S7H, and S7I). Deletion of the TAR-L motif by TALEN also significantly diminished the viability of C4-2 cells (Figure 6F), and this result was consistent with the finding that TAR-L deletion significantly decreased expression of the *trans* targets that are important for cell-cycle progression, proliferation, and survival (Figure S7A).

DISCUSSION

Activation of the P-TEFb complex is important for Pol II-Ser2p, release of paused Pol II, and gene transcription, but the underlying mechanisms are not fully understood. An essential step in P-TEFb activation is its dissociation from the inhibitory subunit composed of 7SK snRNA and HEXIM1/2 (Liu et al., 2013; Nguyen et al., 2001; Yang et al., 2001). HIV-1 Tat (via interaction with TAR RNA) and BRD4 (via interaction with JMJD6) are capable of extracting P-TEFb out from the inhibitory 7SK/HEXIM1/2 complex by competing away and mediating the decapping/demethylation of 7SK, respectively (Egloff et al., 2006; Liu et al., 2013; Roy et al., 1990). We identified a TAR-L sequence in *PSA* eRNA and other AR-eRNAs that share a similar secondary structure with the 3′ end of 7SK snRNA and HIV-1 TAR RNA. We provided evidence that *PSA* eRNA binds to CYCLIN T1, but not HEXIM1, and that the binding is mediated by the TAR-L motif. Accordingly, we showed that *PSA* eRNA promotes P-TEFb activation as a competitor of 7SK. Through reporter gene and TALEN-based gene editing assays, we provided in vitro and in vivo evidence that the TAR-L motif in *PSA* eRNA is important for P-TEFb activation and Pol II-Ser2p. Thus, our findings reveal a previously undefined mechanism of P-TEFb activation and a function of eRNA in regulating P-TEFb activation, Pol II-Ser2p, and gene transcription in *cis* and in *trans* (Figure 6G). It is worth noting that HIV-1 Tat forms a complex with TAR RNA to activate P-TEFb by competing away the inhibitory subunit 7SK and HEXIM1/2 (Zhou et al., 2012). At present, however, whether *PSA* eRNA also requires a similar partner activator to activate the P-TEFb complex is unclear, and further investigation is warranted.

Reactivation of AR is a central mechanism for CRPC progression. Targeting the androgen-AR axis by the second-generation endocrine therapies (e.g., abiraterone and enzalutamide) has been effective for CRPC treatment in clinic. However, drug resistance ultimately develops in some patients, presumably due to the expression of *AR* splice variants and/or other undefined mechanisms (Antonarakis et al., 2014). Enhancer functions are important for tissue-specific gene expression and cell-lineage development (Maston et al., 2006; Ørom and Shiekhattar, 2013; Xie and Ren, 2013). A recent seminal finding is that enhancers make eRNAs. In the present study, eRNA profiling analysis has allowed us to define a signature set of AR-regulated genes whose expression associates with CRPC progression in patients. These findings imply that aberrant eRNA expression represents a new gauge of abnormal AR activity in CRPC. We further revealed that, through both the *cis* and *trans* mechanisms of action, *PSA* eRNA plays a key role in increasing aberrant AR activity and CRPC cell viability. These results highlight a possibility that effective targeting of *PSA* eRNA, alone or in combination with other anticancer agents such as the antiandrogens bicalutamide or enzalutamide, could serve as a therapeutic option for CRPC treatment in clinic. It is worth noting that, in the present study, we mainly focused on the investigation of the functional importance of *PSA* eRNA. With the unfolding of the function of other AR-eRNAs, it is conceivable that combined knockdown of a set of AR-eRNAs may result in greater inhibition or elimination of CRPC growth. We envision that, although the mechanisms underlying aberrant AR activity in CRPC have not been fully understood, blockage of the activities of key enhancers by targeting functional AR-eRNAs may open up a new avenue for CRPC therapy.

Despite being extensively utilized as a biomarker for prostate cancer diagnosis and prognosis, as well as a model gene to study androgen action, the significance of the *PSA* gene in prostate cancer growth and survival has not been well established. We demonstrated that knockdown of *PSA* eRNA by ASOs largely decreased the viability of C4-2 CRPC cells, implying the significance of *PSA* eRNA in CRPC cell growth and survival. Deletion of the TAR-L motif by TALEN also significantly diminished the viability of C4-2 cells, and this result was consistent with the finding that TAR-L deletion significantly decreased expression of the *trans* targets of *PSA* eRNA that are important for cell-cycle progression, proliferation, and survival. Thus, in addition to the *cis* function in the regulation of *PSA* mRNA expression, we found that *PSA* eRNA can *trans*-regulate expression of a subset of genes involved in androgen action; cell-cycle progression; and cell proliferation, survival, and growth, as well as migration and invasion. Our data reveal that, as one of the genes most strongly transactivated by AR signaling, the *PSA* gene produces eRNAs from its enhancer to "magnify" AR action by *trans*-regulating a large number of downstream target genes. Our findings also suggest that, besides being as a biomarker, the *PSA* gene can produce eRNAs from its enhancer that are functionally important. Thus, *PSA* represents an unconventional class of genes whose tumor biological significance is not solely reflected by their protein function but also by other functional components such as eRNA.

In summary, we identified a TAR-L motif in AR-eRNAs and demonstrated that this motif in *PSA* eRNA is important for CYCLIN T1 binding, P-TEFb activation, Pol II-Ser2p, *cis*- and *trans*-regulation of transcription of target genes, and CRPC cell growth (Figure 6G). Therefore, our findings not only reveal a previously undefined mechanism of action for enhancer RNAs in both *cis*- and *trans*-regulation of gene transcription but also uncover a new mode of P-TEFb activation. These results suggest that targeting AR-eRNAs using new methods such as ASOs represent an alternative arsenal of AR-directed therapies for CRPC.

EXPERIMENTAL PROCEDURES

For further details, see the Supplemental Experimental Procedures.

Cell Lines, Cell Culture, and Reagents

LNCaP, MCF-7, and HepG2 cell lines were purchased from ATCC. The C4-2 cell line was purchased from UroCorporation. Mibolerone and bicalutamide were purchased from Sigma-Aldrich. Enzalutamide was kindly provided by Medivation.

ChIP-Seq, Data Analysis, and ChIP-qPCR

ChIP and ChIP-seq library preparation were performed as described previously (Boyer et al., 2005), and high-throughput sequencing was performed using HiSeq2000 platforms at the Mayo Genome Core. The detail procedures are shown in the Supplemental Information.

Design and Screening of Highly Optimized Generation-2.5 Antisense Oligonucleotides

All antisense oligonucleotides (ASOs) contained a full phosphorothioate backbone. A large number of ASOs targeting sense-strand *PSA* (or *KLK3*) eRNA were screened by Ionis Pharmaceuticals for highly efficient reduction of *PSA* eRNA. The detail procedures are shown in the Supplemental Information.

Transcription Activator-like Effector Nuclease Construction

Transcription activator-like effector nucleases (TALENs) were designed using Mojo Hand software. The detail procedures are shown in the Supplemental Information.

ACCESSION NUMBERS

The accession number for the sequencing datasets reported in this paper is GEO: GSE55032.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.03.038.

AUTHOR CONTRIBUTIONS

H.H. and Y.Z. conceived the study, and H.H. and Y.S. supervised the study. Y.Z. performed most of the experiments, with help from S.R., Lan Wang, P.R.B., M.S.M., X.G., M.Q., S.C.E., and T.K., and Liguo Wang performed bioinformatics and statistics analyses. S.R., R.L.V., M.K., D.J.T., J.Z., R.J.K., and Y.S. contributed to acquisition and analysis of PDX and patient samples. Y.K. and R.M. supervised ASO screening.

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