

Enhancer RNAs participate in androgen receptor-driven looping that selectively enhances gene activation

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The androgen receptor (AR) is a key factor that regulates the behavior and fate of prostate cancer cells. The AR-regulated network is activated when AR binds enhancer elements and modulates specific enhancer-promoter looping. Kallikrein-related peptidase 3 (KLK3), which codes for prostate-specific antigen (PSA), is a well-known AR-regulated gene and its upstream enhancers produce bidirectional enhancer RNAs (eRNAs), termed KLK3e. Here, we demonstrate that KLK3e facilitates the spatial interaction of the KLK3 enhancer and the KLK2 promoter and enhances long-distance KLK2 transcriptional activation. KLK3e carries the core enhancer element derived from the androgen response element III (ARE III), which is required for the interaction of AR and Mediator 1 (Med1). Furthermore, we show that KLK3e processes RNA-dependent enhancer activity depending on the integrity of core enhancer elements. The transcription of KLK3e was detectable and its expression is significantly correlated with KLK3 ($R^2 = 0.6213$, $P < 5 \times 10^{-11}$) and KLK2 ($R^2 = 0.5893$, $P < 5 \times 10^{-10}$) in human prostate tissues. Interestingly, RNAi silencing of KLK3e resulted in a modest negative effect on prostate cancer cell proliferation. Accordingly, we report that an androgen-induced eRNA scaffolds the AR-associated protein complex that modulates chromosomal architecture and selectively enhances AR-dependent gene expression.

KLK3e/AR/Med1 complex | chromosomal looping

Androgens and the androgen receptor (AR) play pivotal roles not only in the development of the normal prostate gland but also in the growth and progression of prostate cancer (PCa) (1–4). Androgens exert their biological and physiological effects through activating AR transcriptional activity. AR, a transcription factor, recruits ligand-dependent transcriptional machinery that activates a tissue-specific transcriptional program involved in development and differentiation (4–6). In PCa cells, AR occupies numerous gene loci to activate transcripts mediating critical cellular activities, such as energy and metabolism (5). AR activation and reactivation are essential for PCa development and tumor recurrence (7).

One unanticipated observation that emerged from multiple global studies of RNA polymerase II (Pol II) occupancy was that Pol II binds to a large number of intergenic AR-bound enhancers marked by histone H3 lysine 4 monomethylation (H3K4me1) and lysine 27 acetylation (H3K27ac) and produces enhancer-derived long noncoding RNAs (eRNAs) (8–11). Although eRNAs are bidirectional and nonpolyadenylated, their induction correlates with the induction of adjacent exon-coding genes (9). The activity of eRNAs was shown to augment the expression of not only neighboring but also intrachromosomally distant genes to convey enhancer-dependent transcription (12, 13). Recently, the 5'-GRO-seq data have revealed that sequence-specific transcription factors and eRNA transcripts are required for transfer of full enhancer activity to a target promoter (14).

Moreover, eRNAs have been shown to configure a chromatin state that facilitate transcription factors binding on the target promoters at defined genomic loci (15).

Mechanistically, eRNAs have been shown to associate with the cohesin complex at estrogen receptor binding sites and contribute to gene activation by stabilizing estrogen receptor- α (ER- α)/eRNA-induced enhancer-promoter looping (16, 17). In addition, ncRNA-activating (ncRNA-a), a class of enhancer-like RNAs, has been shown to interact with the Mediator complex that enhances transcription in a *cis*-mediated mechanism (18), highlighting an RNA-dependent functional effect. Therefore, we hypothesized that eRNAs facilitates spatial communication of enhancers and promoters, thereby enhancing transcriptional activation events. Here, we report that the Kallikrein-related peptidase 3 (KLK3) enhancer, one of the strongest AR-bound enhancers in prostate cancer cells, produces KLK3 eRNA (KLK3e), which impacts androgen-induced gene activation. This work reveals that KLK3e selectively enhances AR-regulated gene expression through its collaboration with the AR-dependent looping complex. Thus, our findings provide evidence for a positive regulatory loop in which the AR-regulated network is modulated by an intermediate eRNA.

Results

The KLK3 Enhancer Produces eRNA (KLK3e). We compiled recently established genomic datasets (19) and showed that the KLK3

Significance

We report that enhancer RNAs (eRNAs), a class of long non-coding RNAs, participate in the androgen receptor (AR)-dependent looping complex that enhances spatial communication of distal enhancers and target promoters, leading to transcriptional activation events. Furthermore, our data show that KLK3 eRNA (KLK3e) selectively enhances the gene expression of AR-regulated genes, and provide evidence for a positive regulatory loop in which AR-dependent transcription is modulated by an intermediate eRNA. These findings may translate into improved RNA-based therapy (eRNA suppression) to enhance the durability of androgen deprivation therapy (ADT) and prediction of the efficacy of ADT by measuring the enhancer-derived activity (eRNA expression) in prostate tumors.

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enhancer, also defined as the androgen response element III (ARE III) (20), was marked by AR, H3K27ac, and H3K4me1, implying that this region is transcriptionally active (Fig. 1A). In fact, the transcription of KLK3 eRNA (KLK3e) was demonstrated by GRO-seq data (10) and confirmed by our reverse transcription–quantitative PCR (RT-qPCR) analyses in androgen-dependent LNCaP and VCaP, and androgen-independent LNCaP-abl cells (21) (Fig. 1B and Fig. S1A and B). The expression of KLK3e was induced within the first hour of dihydrotestosterone (DHT) exposure and its expression level gradually increased in a time-dependent manner, resembling the expression of mature KLK3 in both LNCaP and VCaP cells; in contrast, although transcription at the KLK3 locus remains active in androgen-independent LNCaP-abl, androgens (DHT) did not stimulate their mRNA expression to a comparable magnitude as those in parental LNCaP cells (Fig. S1B). Next, we investigated the impact of AR antagonist, bicalutamide, on the expression of KLK3e. The expression of KLK3e and KLK3 was induced upon DHT stimulation and blocked by bicalutamide (Fig. S1C), revealing that ligand-dependent eRNA synthesis takes place in association with target mRNA transcription. Taken together, our findings are consistent with the previous report that there is a high correlation of activity-dependent induction between eRNAs and adjacent coding mRNAs (9).

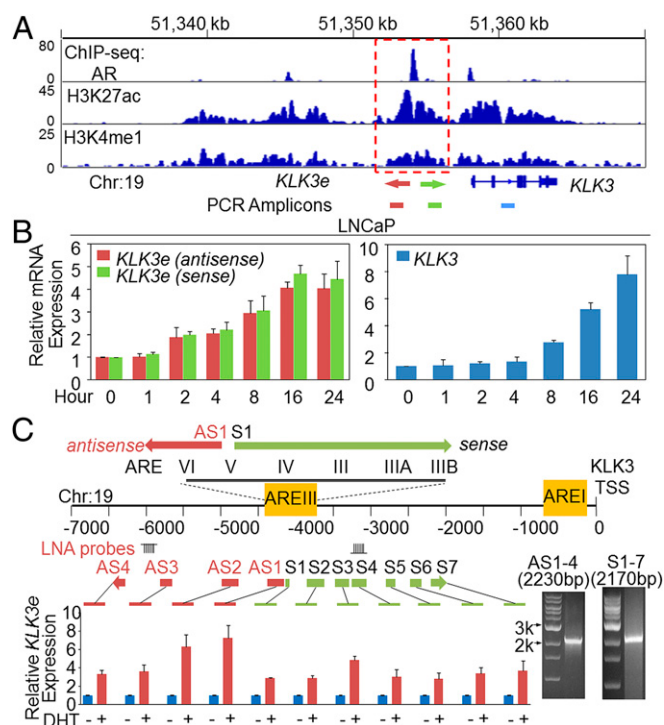
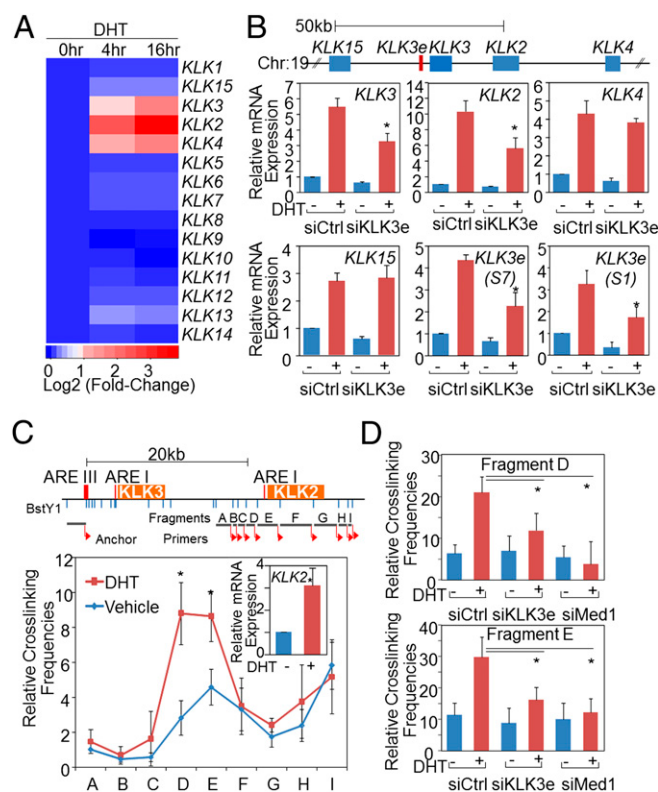


Fig. 1. The KLK3 enhancer produces lncRNAs. (A) The chromatin status in the vicinity of KLK3. ChIP-seq datasets of AR, H3K27ac, and H3K4me1 (19). (B) RT-qPCR analyses of expression of antisense KLK3 eRNA (red bar), sense KLK3 eRNA (green bar), and mature KLK3 (blue bar) upon DHT (10 nM) treatment in a time-dependent manner in LNCaP cells. (C, Upper) A diagram of functional ARE positions that are relative to the KLK3 transcription starting site (TSS) adapted from ref. 20. (Lower) RT-qPCR analyses of the expression of segments of KLK3e with DHT (10 nM) treatment in LNCaP cells. The regions of LNA probes against the antisense and sense KLK3e were as indicated. Primer sets for qPCR were designed to specifically target either antisense (primers AS1–AS4) or sense strand (primer S1–S7) of KLK3e as shown. The right electrophoresis images show the PCR products of antisense (2,230 bp) and sense (2,170 bp) KLK3e. All data analyzed by RT-qPCR were normalized to the expression level in vehicle (–). Data are shown as mean $\pm$ SD ($n \geq 3$).

Although multiple studies have identified different eRNA species from mammalian enhancers, the precise mapping of eRNAs has not been described. Because eRNAs produced from these intergenic enhancers are low at steady state (9) and estimated at fewer than 100 copies per cell (17), we enriched cellular RNAs by depleting rRNAs before reverse transcription. PCR primer sets against the antisense (AS1–AS4) and sense (S1–S7) of KLK3e were designed based on the GRO-seq and genomic datasets (10, 19). As shown in Fig. 1C, the transcription of bidirectional KLK3e was captured; whereas, no robust DHT-induced transcription was detectable beyond AS4 and S7. We performed primer-extension PCR assay to connect shorter fragments (Fig. S1D) and further showed PCR products for the antisense (2,230 bp) and sense (2,170 bp) of KLK3e (Fig. 1C and cDNA sequences available in *SI Experimental Procedures*). To distinguish whether the eRNA at KLK3 enhancer are polyadenylated or not, we performed reverse transcription using random hexamer primers and 3'-RACE (poly-T) adapter from the same RNA materials. As shown in Fig. S1E, the expression of AS4 and S7 was substantially induced by DHT treatment, suggesting that the poly-A tail was added to the 3'-end of antisense and sense KLK3e. In contrast, S1 showed less DHT-stimulated expression in the poly-T priming compared with random hexamer primed cDNA. These data suggest that this locus produces a mixture of RNA species; some of them are polyadenylated, whereas others may be actively undergoing synthesis of polyadenylation, but we cannot totally exclude the existence of those species without poly-A tails. To detect the size of the mature eRNA at this loci, we customized double DIG-labeled probes using locked nucleic acids (LNA) (Exiqon). Northern analysis revealed that a major form (~ 2.5 kb) and a minor form (~ 1.7 kb) of sense KLK3e were produced upon DHT induction, whereas no signal was detected at the antisense strand (Fig. S1F), suggesting that the expression of antisense strand may be lower than the levels that can be detected by the LNA probe (Fig. S1F). Collectively, this suggests that the sense strand (S1–S7) KLK3e, a ~ 2.2 -kb transcript, is polyadenylated and yields a ~ 2.5 -kb transcript shown in Northern analysis. Although the antisense KLK3e can be polyadenylated and detected by qPCR, its expression may be substantially lower than the sense strand and cannot be detected by Northern analysis under the same experimental settings, indicating that the sense strand KLK3e is the dominant species.

The Regulatory Function of KLK3e on the Kallikrein Locus. To investigate the potential function of eRNAs in transcriptional regulation, short interfering RNAs (siRNAs) targeting KLK3e (siKLK3e) were designed on the basis of KLK3e mapping. Results obtained from distinct sets of siRNAs against the sense strand of KLK3e showed that siKLK3e specifically targeted sense KLK3e and significantly impeded DHT-induced KLK3 expression in both LNCaP and VCaP cells but had a minimal impact on LNCaP-abl cells (Fig. 2B and Fig. S2A–E), consistent with the notion that ligand-activated eRNAs are important for nearby gene activation (13, 17). Considering that KLK15, 2, and 4 were adjacent to KLK3 and androgen-responsive (Fig. 2A), we examined whether KLK3e impacted transcription of these KLKs. Interestingly, knocking down of sense KLK3e significantly inhibited the expression of KLK2, whereas other KLKs remained unchanged (Fig. 2B). We also investigated the effect of antisense KLK3e on the regulation of target gene expression. RNAi silencing of the antisense of KLK3e showed a marginal inhibitory effect on KLK3 and KLK2 gene expression (Fig. S2F). Despite the presence of bidirectional KLK3e transcription, data from siRNA to KLK3e and Northern analysis showed that the sense strand of KLK3e is the dominant transcript with functional effects on the expression of KLK3 and distant KLK2. Thus, we chose the sense KLK3e and continued to study its RNA-dependent enhancer activity.

Because the induction of eRNA is strongly correlated with the enhancer looping to target gene promoters (13, 16, 17), it is



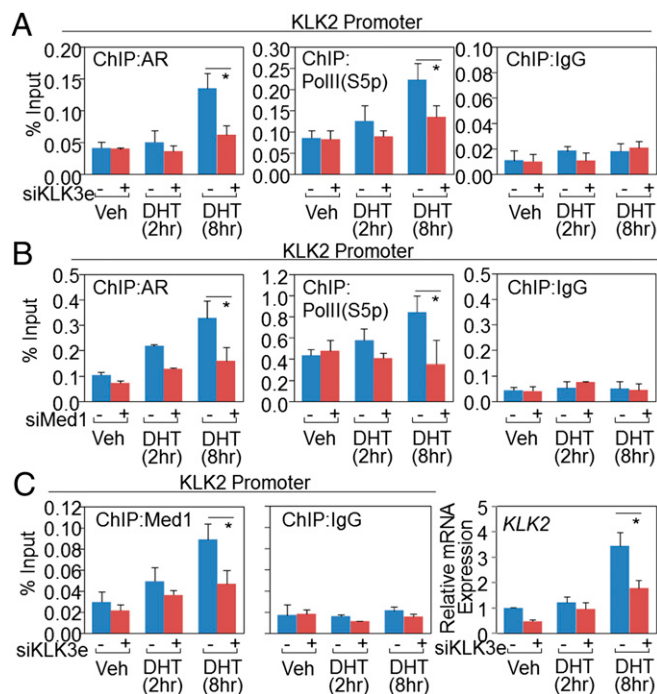


Fig. 3. The KLK3e/AR/Med1 ribonucleoprotein complex transcriptionally regulates target promoters. Chromatin immunoprecipitation (ChIP) of AR or Med1 or activated Pol II (S5p) was performed in KLK3e- or Med1-depleted LNCaP cells with or without DHT (10 nM) for 2 or 8 h. (A–C) qPCR was conducted to measure the action of AR, Pol II (S5p), and Med1 at the KLK2 promoter. Data are shown as mean $\pm$ SD ($n \geq 3$) and $*P < 0.05$.

The expression of the KLK3e from the luciferase reporter was validated by RT-PCR (Fig. S4A), indicating that AREs have the capacity to produce eRNAs. We next asked whether the KLK3e transcript alone is adequate for this transcriptional enhancement. For this purpose, we performed a cotransfection study based on reporters together with CMV-driven sense KLK3e constructs. As shown, the full-length (S1–S7) and flipped (S7–S1) KLK3e increased luciferase expression by 2- to 2.5-fold, whereas ARE deletion (S2–S7) failed to enhance promoter activity (Fig. 4B). Furthermore, antisense KLK3e (AS1–AS4) was unable to induce luciferase expression as sense KLK3e (S1–S7) did (Fig. S4B and C), again arguing that the sense KLK3e is the functional long noncoding RNA (lncRNA) rather than the antisense strand. To examine whether AR is required for the action of KLK3e, we conducted experiments in AR-negative COS-7 cells and confirmed that the activity of KLK3e depends on the availability of ligand-activated AR (Fig. S4D and E). Altogether, these data show that the core enhancer elements are essential for the enhancer activity of KLK3e and that ectopic KLK3e induces the promoter activity on a luciferase reporter, implying a transregulatory activation mediated by KLK3e.

As a complementary approach, we also addressed whether the observed luciferase expression is mediated through endogenous KLK3e. RNAi silencing of KLK3e significantly reduced luciferase activity in the reporters with or without KLK3e insertion (Fig. 4C, Left), showing that KLK3e is required for the enhancement of DHT-induced activation. When KLK3e was ectopically expressed, it restored the luciferase expression inhibited by KLK3e suppression (Fig. S4F). Most importantly, the luciferase activity in the reporter with KLK3e insertion was reduced by ~50% in Med1-depleted cells (Fig. 4C, Right), suggesting that Med1 facilitates KLK3e-dependent transcription. Notably, ncRNA-a has been shown to mediate long-range transcriptional activation through its association with the Med1 complex (18). In summary, our results

strongly suggest that KLK3e forms a functional complex with AR and Med1 that facilitates the association of AR-bound enhancers with promoters of target genes, resulting in transcriptional activation.

KLK3 eRNA Selectively Enhances AR-Regulated Gene Expression. To explore the potential effect of KLK3e beyond the KLK locus, we measured the expression of canonical androgen-regulated genes in KLK3e-depleted cells. Suppression of KLK3e decreased the expression of NKX3.1, FKBP5, and PLZF, whereas the expression of TMPRSS2, PDE9A, and GUCY1A3 was not affected (Fig. S4A). Additionally, we observed a comparable KLK3e-mediated inhibition of NKX3.1 gene expression in VCaP cells (Fig. S2D). Moreover, we showed the expression eRNAs at upstream of NKX3.1, FKBP5, PLZF was induced by DHT but not affected by KLK3e siRNA knockdown (Fig. S5A), suggesting that the KLK3e siRNA does not interfere with the transcription of eRNAs found at the enhancers of NKX3.1, FKBP5, and PLZF. Although eRNA-mediated *trans* effects are likely to be relatively infrequent or quantitatively small (17), our data show that KLK3e selectively regulates the expression of AR-regulated genes that do not reside on the same chromosome, implying a transregulatory function of KLK3e on AR-dependent gene expression.

We further assessed the regulatory role of KLK3e and Med1 on the promoter of NKX3.1 and TMPRSS2 to mechanistically validate our above findings in the KLK loci. Although depletion of KLK3e significantly reduced the enrichment of AR and Pol II S5p at the NKX3.1 promoter (Fig. S5B, Upper), the action of these factors at the TMPRSS2 promoter was unaffected (Fig. S5B, Lower). In contrast, Med1 knockdown considerably blocked both AR occupancy and Pol II activity at both the NKX3.1 and TMPRSS2 promoters (Fig. S5C). These results demonstrate that KLK3e selectively enhances gene expression, whereas Med1 has a more general effect on the regulation of AR-dependent transcription. Most importantly, KLK3e depletion reduced Med1 recruitment to the promoter of NKX3.1, not TMPRSS2 (Fig. S5D and E), revealing that this eRNA/AR/Med1 complex may

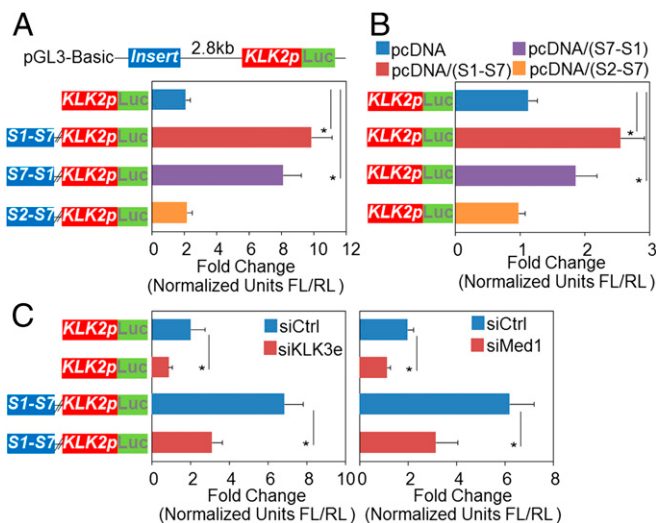


Fig. 4. The core enhancer element renders KLK3e RNA-dependent enhancer activity. (A, Upper) Schematic representation shows the insertion in KLK2 promoter driven luciferase reporter. (Lower) Reporter assay analyses were performed with or without insertion of full-length (S1–S7), flipped (S7–S1), and ARE deleted (S2–S7) of sense KLK3e. (B) Reporter analysis of cotransfecting the reporter with independent overexpressing vector as indicated. (C) The effects of siKLK3e (Left) or siMed1 (Right) on the KLK2 promoter-driven Luciferase activity. Luciferase activity was measured in the presence of DHT (10 nM) for 24 h. All data shown are mean $\pm$ SD of at least three independent experiments. $*P < 0.05$; firefly luciferase/*Renilla* luciferase (FL/RL).

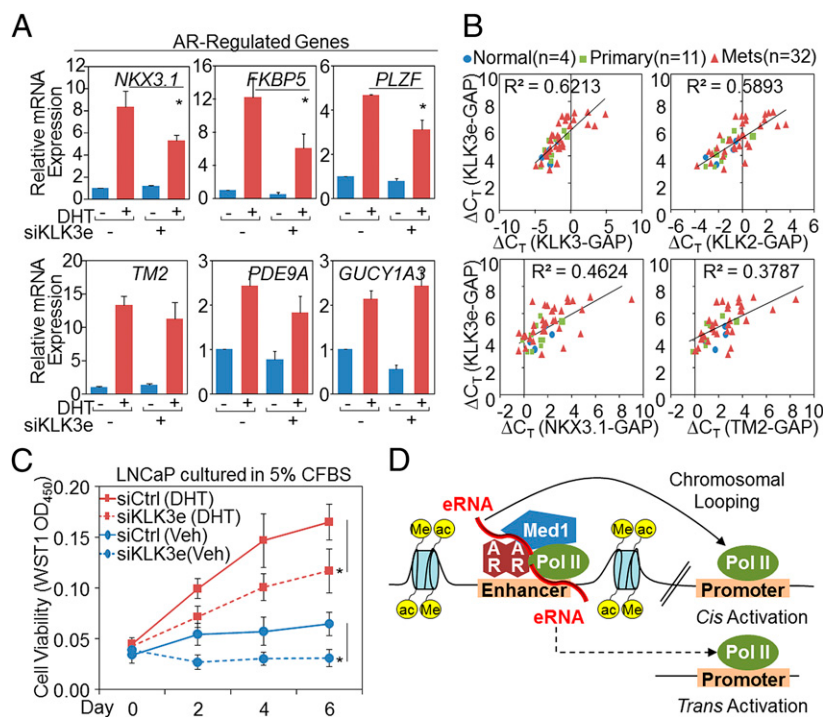


Fig. 5. KLK3e selectively enhances AR-regulated gene expression. (A) LNCaP cells were treated with vehicle or DHT (10 nM) and/or siCtrl or siKLK3e. The effect of siKLK3e on AR-regulated gene expression was determined by RT-qPCR. (B) Correlation between KLK3e (S1) and target gene expression in vivo. All data were normalized for GAPDH by the differences in threshold cycles (ΔC_T) from the normal human prostate gland ($n = 4$), primary ($n = 11$) and metastatic ($n = 32$) prostate tumors (25). (C) LNCaP cells were cultured in 5% charcoal/dextran-treated FBS (C-FBS) medium, followed by siRNA knockdown of scrambled control (siCtrl) or KLK3e (siKLK3e) with ethanol (veh) or DHT (10 nM) treatment. Cell growth was measured by WST1 assay. Data represent mean $\pm$ SD ($n \geq 3$), and $*P < 0.05$. (D) A proposed model for functional activities of the eRNA/AR/Med1 complex in DHT-induced *cis* (solid line) and potential *trans* (dashed line) gene activation events.

possess a degree of specificity in the AR-dependent transcriptional machinery. Furthermore, we evaluated the gene expression from the normal human prostate gland ($n = 4$), primary ($n = 11$) and metastatic ($n = 32$) human prostate tumors (25), and showed that the correlation (R^2) of KLK3e (S1) with KLK3, KLK2, NKX3.1, and TMPRSS2 was 0.6213 ($P < 5 \times 10^{-11}$), 0.5893 ($P < 5 \times 10^{-10}$), 0.4624 ($P < 2 \times 10^{-7}$), and 0.3787 ($P < 5 \times 10^{-6}$), respectively. In addition, the expression of the KLK3 and KLK2 was the most correlated ($R^2 = 0.8361$, $P < 3 \times 10^{-16}$) (Fig. 5B and Fig. S5F). Collectively, these in vivo data indicate that the transcription of KLK3e was detectable and its presence correlates with KLK3/2 gene expression. We noted less of a correlation between KLK3e expression and those genes *in trans*, and the correlation was stronger with NKX3.1 than TMPRSS2, consistent with our in vitro data, although these data do not prove a direct effect in vivo of KLK3e on the transcription of these genes. Next, we investigated the biological effect of KLK3e siRNA on LNCaP cells and showed that KLK3e depletion significantly compromised cell growth in the presence and absence of exogenous androgens (Fig. 5C). Although this negative effect is modest, we postulate that suppression of multiple eRNAs produced from distinct enhancer elements may synergistically block the AR transcriptional program. Thus, substantial efforts are needed to fully characterize the transcriptional and biological functions of other significant androgen-induced eRNAs in the future.

Discussion

We demonstrate that bidirectional KLK3e was produced from one of the strongest AR-bound enhancers (ARE III) and its expression pattern resembles KLK3 in prostate cancer cell lines (Fig. 1 and Fig. S1), in agreement with the previous report that a high correlation of activity-dependent induction exists between eRNAs and adjacent coding mRNAs (9). Suppression of KLK3e exclusively reduces gene expression of KLK3 and KLK2, not other KLK family members and that KLK3e facilitates the assembly of the transcriptional apparatus at the promoters during gene activation events. This classifies KLK3e as a family of functional lncRNA involved in enhancing long-distance gene transcription.

Whether eRNAs function as molecular bridges that mediate spatial interactions of distal enhancers and target promoters, or directly configure a chromatin state that facilitate transcription factor binding at the target promoters is unclear. Recently, chromatin isolation by RNA purification coupled with deep sequencing was deployed to interrogate the nature of RNA and DNA interaction in a genome-wide scale (26). Rosenfeld and colleagues (17) used this methodology to identify potential sites where estrogen-induced FOXC1 eRNA localizes in the genome. Although FOXC1 eRNA is specifically enriched in a small fraction of genomic loci, there were no known estrogen-regulated genes found adjacent to these RNA/DNA binding sites (17), implying that the functional effect of eRNAs on target genes may not completely rely on nucleotide base pairing. In this report, we demonstrate that KLK3e mediates chromosomal looping of KLK3/2 loci and confirmed in a heterologous reporter assay that KLK3e cooperates with AR and Med1 to enhance KLK2 promoter activity through the core enhancer element (ARE III). The state of KLK3e and KLK3/2 was further validated in human prostate tissues ($n = 47$) and showed that the expression of KLK3e significantly correlates with KLK3 ($R^2 = 0.6213$, $p < 5 \times 10^{-11}$) and KLK2 ($R^2 = 0.5893$, $p < 5 \times 10^{-10}$), implying that KLK3e may coordinate the transcription of KLK3/2 (Fig. 5B). However, we do not completely understand how KLK-associated eRNAs are regulated in castration-resistant prostate cancer (CRPC), although it is well accepted that the AR-mediated transcriptional program remains active. This observation can be explained by the activity of other nuclear receptors, because the core sequence of ARE can be also be recognized by glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR) (27). More importantly, it has been shown that AR inhibition resulted in GR up-regulation in a subset of prostate cancer cells supporting a mechanism of bypassing AR blockade via an alternative nuclear receptor in CRPC (28). Thus, it is possible that GR, PR, or MR may mediate the expression of KLK3e, KLK3, and KLK2, attributing to adaptive or compensatory effects generated during CRPC development.

In view of the regulatory potential of lncRNAs on gene regulation *in trans* (29–31), eRNAs may be part of coactivator complexes

and regulatory factors that participate in the transcriptional regulatory network. Although the entire circuitry of AR-dependent genes is unlikely controlled by a single eRNA, presumably, there are more androgen-induced eRNAs, and there could be redundancy in their enhancer activity. It is conceivable that multiple eRNAs produced from distinct enhancer elements tether with their own transcriptional modules that operate simultaneously to initiate an accurate and a rapid transcriptional program. Therefore, we propose that an eRNA may function as a scaffold that guides an AR-associated protein complex to target chromatin and selectively enhances DHT-stimulated transcription either intrachromosomally (*cis* activity) or interchromosomally (*trans* activity). This positive regulatory loop may provide new insight into RNA-dependent functional effects on the regulation of lineage- or tissue-specific gene expression.

Although androgen deprivation therapy (ADT) is designed to block the androgen signaling pathway and is the standard treatment for advanced prostate cancer, this study may help us to understand how androgen ADT influences the activity of AR-bound enhancers through altered expression of eRNAs. In addition, the differential eRNA expression patterns observed may be predictive markers of ADT efficacy for individual prostate cancer patients. More importantly, eRNAs may represent new targets that when suppressed may improve the therapeutic durability of ADT.

Experimental Procedures

ChIP Assay. ChIP experiments were performed as previously described (19) with some modifications. Briefly, 10 million cells were used per IP. Cells were fixed with 1% formaldehyde solution. DNA was sonicated and subjected to

immunoprecipitation with antibodies against androgen receptor (N-20X; Santa Cruz Biotechnology), Med1/TRAP220 (A300-793A; Bethyl Laboratories), RNA Polymerase II (p-Ser5) (ab5131; Abcam), or nonspecific IgG. DNA was purified and analyzed by qPCR. The primers for qPCR are provided in [SI Experimental Procedures](#).

RIP Assay. RIP experiments were performed as described previously with some modification (31). Briefly, 10 million cells were used per IP. Cells were fixed in 0.3% formaldehyde solution. Total chromatin and RNAs were sonicated and subjected to immunoprecipitation with the same AR and Med1 antibodies used in ChIP experiments. Immunoprecipitated RNAs were isolated using TRIzol LS reagent, followed by cDNA synthesis. The primers for qPCR are provided in [SI Experimental Procedures](#).

3C Assay. The 3C assay was performed as previously described with some modifications (32). After DHT (10 nM) treatment, LNCaP cells were fixed with 1% formaldehyde. Cell pellets were lysed and resuspended in restriction buffer for BstY1 and 0.1% SDS for 10 min at 65 °C. Triton X-100 was added to a final concentration of 1.8% followed by overnight digestion of BstY1 (1,500 U per 10⁷ cells) at 37 °C. DNA ligation was performed for 4 d at 16 °C. The ligated samples were reverse cross-linked with proteinase K at 65 °C overnight, followed by phenol/chloroform extraction and EtOH precipitation. The primers for qPCR are provided in [SI Experimental Procedures](#).

Additional experimental procedures and methods are listed in [SI Experimental Procedures](#).

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Supporting Information

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SI Experimental Procedures

Cell Culture and siRNA Transfection. LNCaP cells were cultured in RPMI 1640 medium with 10% FBS. VCaP and COS-7 cells were cultured in DMEM with 10% FBS. Before androgen induction or transfection, cells were grown to 50–60% confluence in 5% charcoal/dextran-treated FBS (C-FBS) medium for 3 d and then treated with 10 nM dihydrotestosterone (DHT) or siRNA (50 nM).

RNA, Isolation, Purification, and cDNA Synthesis. Prostate cancer cells and human prostate tissue frozen sections (1) were harvested and resuspended in TRIzol (Invitrogen), and RNAs were extracted according to the manufacturer's protocol. Total RNAs were purified by RNeasy Kit (QIAGEN). Purified RNA from LNCaP cells was subsequently washed by RiboMinus Eukaryote Kit (Invitrogen). The purified RNA fraction was subjected to cDNA synthesis, which was performed using High Capacity cDNA Reverse Transcription Kit (Life Technology) or 3'-RACE poly-T adaptor (Ambion).

Reagents Used for siRNA-Mediated Knockdown Experiments. siRNA sequences targeting the sense strand of KLK3e were as follows: 3'-end (S7) #1, 5'-gaggaugaguugaggauuuu-3'; #2, 5'-ggguuggcuuguuagguuuu-3'; 5'-end (S1), 5'-ggaacauuuguaucgaaauu-3', and the antisense strand (siAS), 5'-gcaaaaggauacagaugaaau-3'. siRNA targeting Med1 and nontargeting siRNA were ON-TARGETplus Human MED1 siRNA and ON-TARGETplus Non-Targeting siRNA#1 from Thermo Scientific. Transfection of LNCaP and COS-7 cells was performed with Lipofectamine 2000 or Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol.

Plasmid Constructs for Luciferase Assay. Full-length (S1–S7), flipped (S7–S1), ARE-deleted (S2–S7), and antisense (AS1–AS4) of KLK3e were subcloned into pcDNA3 (Invitrogen) using EcoRI and XbaI sites. pGL3-Basic (Promega) was digested with SacI and XhoI, and KLK2 promoter (–407 ~+143) promoter was inserted into these sites. Additionally, TK promoter sequences from pRL-TK (Promega) were subcloned into pGL3/KLK2 promoter using BglII and HindIII. Full-length (S1–S7), flipped (S7–S1), and ARE-deleted (S2–S7) KLK3e were inserted into downstream of SV40 late poly(A) signal of luciferase reporter. Luciferase experiments were conducted in 96-well white plates using Dual-Glo (Promega) according to the manufacturer's protocol.

Northern Blot Analysis. Northern blot assay was performed using DIG Northern Starter Kit (Roche) with some modifications. Total RNA was isolated and purified from LNCaP treated with or without DHT (10 nM). Two or 40 µg of RNA were subjected to electrophoresis, followed by hybridization using the 10 nM LNA probes (Exiqon) against KLK3: /5DigN/tcaggagctcatatcgtaga/3DigN/; actin (Exiqon); sense KLK3e: /5DigN/atgctggatgatgagtgatga/3DigN/; and antisense KLK3e: /5DigN/agtacctaactctctga/3DigN/.

Cell Proliferation Assay. Cell growth was examined using the WST1 assay (Roche) following the manufacturer's protocol.

Briefly, 3,000 LNCaP cells were grown in a 96-well plate in the RPMI 1640 medium containing 5% C-FBS. After 2-d incubation, cells were transfected with 50 nM siRNAs to nonsilencing control (siCtrl) or KLK3e (siKLK3e). After 24 h of incubation, cells were treated with ethanol (Veh) or DHT (10 nM). Then, the WST1 assay was performed after days 0–6.

Sense and Antisense KLK3e Sequences. *Sense (S1–S7).* TGGGA-CAACTTGCAAACCTGCTCAGCCTTTGTCTCTGATGAAG-ATATTATCTTCATGATCTTGGATTGAAAACAGACCTAC-TCTGGAGGAACATATTGTATCGATTGCTCTTGACAGTA-AACAAATCTGTTGTAAGAGACATTATCTTTATTATCTA-GGACAGTAAGCAAGCCTGGATCTGAGAGAGATATCAT-CTTGCAAGGATGCCTGCTTTACAAACATCCTTGAAACA-ACAATCCAGAAAAAAGGTGTTGCTGTCTTTGCTC-AGAAGACACACAGATACGTGACAGAACCATTGGAGAA-TTGCCCTCCCAACACTGTTTCAGCCAGACCTCCACCCT-TGTCTGCAGGACAGTCTCAACGTTCCACCATTAAATA-CTTCTTCTGTACATCCTGCTTATTTATGCCTAACCAAGGTTCTAGGTCCCGATCGACTGTGTCTGGCAGCACT-CCACTGCCAAACCCAGAATAAGGCAGCGCTCAGGA-TCCCGAAGGGGCATGGCTGGGGATCAGAACTTCTG-GGTTTGAGTGAGGAGTGGGTCCACCCTTGAATTTCAAAGGAGGAAGAGGCTGGATGTGAAGGAACTGGG-GGAGGGAAAGTGTGAGTTCCGAACTCTTAGGTCAAT-GAGGGAGGAGACTGGTAAGGTCCCAGCTCCCAGG-TACTGATGTGGGAATGGCCTAAGAATCTCATATCCT-CAGGAAGAAGGTGCTGGAATCCTGAGGGGTAGAGT-TCTGGGTATATTTGTGGCTTAAGGCTCTTTGGCCCT-GAAGGGCAGAGGCTGGAACCTTAGGTCCAGGGTT-TGGGGTGATAGTAATGGGATCTCTTGATTCTCAAG-AGTCTGAGGATCGAGGGTTGCCATTCTTCCATCTT-GCCACCTAATCCTTACTCCACTTGAGGGTATCACCAGCC-CTTCTAGCTCCATGAAGGTGCCCTGGGCAAGCACAAT-CTGAGCATGAAAGATGCCCCAGAGGCCTTGGGTGTCAT-CCACTCATCATCCAGCATCCACACTTGAGGGTGTGGCC-AGCACCATGACGTGATGTTGCTGTGACTATCCCTGCAG-CGTGCCTCTCCAGCCACCTGCCAACCGTAGAGCTGCCG-ACATCCTCCTCTGGTGGGAGTGGCCTGCATGGTGCCAG-GCTGAGGCCTAGTGTGACAGAGGAGCCTGGAATCA-TAGGGATCCAGGACTCAAAAGTGCTAGAGAATGGCC-ATATGTCAACCATCCATGAAATCTCAAGGGCTTCTGGG-TGGAGGGACAGGGCACTGAACTTATGGGTTTTCCT-CAAGTCTATTGCTCTCCCAAGTGTGACTCTCCAGTAC-GAGGCACTGTGCCAGCATCAGCCTTATCTCCACCAC-ATCTTGTAAGGAGGACTACCCAGGGCCCTGATGAA-CACCATGGTGTGTACAGGAGTAGGGGGTGGAGGC-ACGGACTCCTGTGAGGTACAGCCAAGGGAGCATC-ATCATGGGTGGGGAGGAGGCAATGGACAGGCTTG-AGAACGGGATGTGGTGTATTTGTTTCTTCTTGTT-AGATAAAGTGCTGGGTATAGGATTGAGATTGGAGT-ATGAAGACCAGTTAGGATGGAGGATCAGATTGGAG-TTGGGTTAGAGATGGGGTAAATTTGTGCTTCGGAT-GAGTTTGGGATTGACACTGTGGAGGTGGTTTGGGATG-GCATGGCTTTGGGATGGAAATAGATTGTTTTGATGTT-GGCTCAGACATCCTTGGGGATTGAACTGGGATGAAG-CTGGGTTGATTTTGGAGGTAGAAGACGTGGAAGTAG-CTGTCAGATTTGACAGTGGCCATGAGTTTTGTTTGATG-GGGAATCAAACAATGGGGGAAGACATAAGGGTTGGCT-TGTTAGGTTAAGTTGCGTTGGGTTGATGGGGTCGGGGC-TGTGTATAATGCAGTTGGATTGGTTTGTATTAATTTG-GTTTGGGTCAGGTTTTGGTTGAGGATGAGTTGAGGAT-ATGCTTGGGACACCGGATCCATGAGGTTCTCACTGG-AGTGGAGACAAACTTCTTTCCAGGATGAATCCGGG-AAGCCTTAATTCACGTGTAGGGGAGGTCAGGCCACTG-GCTAAGTATATCCTTCCACTCCAGCTCTAAGATGGTCT-TAAATTGTGATTATCTATATCCACCTCTGTCTCCCTCA.

Antisense (AS1–AS4). TCCGGAACACAGATCTGACATGCTCA-TCTGCTGCGTGGGGTTTACTATCTGCCTTGAATCCTAA-

AGTCTGCCCTGAGGAGTCTGGAATCTGGAATCTATT-
ATCTTTAGTTGAACACCTGCAGTCTGCGTTCTGCTTTG-
CAGAGCTGGATTCTTTACCCGTGATCCGTTCTGGCATT-
TCAACTTCACACAGAATAGGCCACTTCATGGTACTGTT-
TTCAACCAATCAAGCAAATTAATCGTTAGAGCCCTTTC-
TAATTCCTCTAGTTGCAATGTTAAACCAATATTTCTG-
TGTAGTGGGTCCATATTAATAAATTCAGCCTGATCTAA-
CCTTATATTCCTTCCACCATTATTCATACCAACCCTT-
AATATGCATTCCACATATATTTCTGGATTCTGTCT-
TATATAATGGGCAATCCCGGCTTTGCCAGTTTCTAGT-
GACCTAACATCTCTGTGACTCATGTTATTGTACTTTAA-
AGTGGAATAATAACAGTTTCTGTACAGTTTAGGGCT-
TCCCAAGATGACCCTCCATTGACATCAGTTGAGATT-
CAGGAGAGTCCAAAAGATGATCCCTTAAGGTTCAGTC-
GTTTGCTAGGATGCTCATGGTTGTGGTTTATTACAGCA-
AAAGGATACAGATGAAAATCTGGCAAGGGAGAGGTG-
AATGAGGCAAAGACAAGGAGAACTCAAAGTGCGAAG-
CTTCTAGTTTTCTTTTCCCGGTGACATCGTGGAAGCA-
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TCCAGAGATTTTTGTGTTTTTTCTGAGACTGAGTCTC-
GCTCTGTGCCAGGCTGGAGTGCAGTGGTGAACCTT-
GGCTCACTGCAAGCTCCGCTCCTGGGTTACGCCATT-
CTCCTGCCTCAGCCTCCTGAGTAGCTGGGACTACAGG-
CACCCGCCACCACGCCTGGCTAATTTTTTTGTATTTTT-
AGTAGAGATGGGGTTTCACTGTGTTAGCCAGGATGG-
TCTCAGTCTCCTGACCTCGTGATCTGCCACCTTGCC-
TCCCAAAGTGCTGGGATTACAGGCGTGAGCCACTGCG-
CCTGGCCGATATCCAGAGATTTTTTGGGGGGCTCCAT-
CACACAGACATGTTGACTGTCTTCATGGTTGACTTTT-
AGTATCCAGCCCCCTCTAGAAATCTAGCTGATATAGTG-
TGGCTCAAAACCTTCAGCACAAATCACACCGTTAGAC-
TATCTGGTGTGGCCCAACCTTCAGGTGAACAAAGG-
CACTCTAAACTGGCAGGATATTCCAAAGCATTAGAGA-
TGACCTCTTGCAAAGAAAAAGAAATGGAAGAAAAA-
AGAAAGAAAGGAAAAAAGAAAAAAGAGATGA-
CCTCTCAGGCTCTGAGGGGAAACGCCTGAGGTCTTTG-
AGCAAGGTCAGTCTCTGTTGCACAGTCTCCCTCACA-
GGGTCAATTGTGACGATCAATGTGGTCACGTGTATGA-
GGCACCAGACATGCCTGGCTCTGGGGAGTGCCGTGT-
AAGTGTATGCTTGCACTGCTGAATGGCTGGGATGTGT-
CAGGGATTATCTTCAGCACTTACAGATGCTCATCTCA-
TCCTCACAGCATCACTATGGGATGGGTATTACTGGC-
CTCATTGATGGAGAACTGGCTGTGGCTCAGAAAG-
GGGGGACCACTAGACCAGGGACACTCTGGATGCTGG-
GGATCCAGAGACCATGACCATCAACCACTGCAGAG-
GAAATTAATTGTGGCTGATGTCCCTGTCTCTGGAGA-
GGGTGGAGGTGGACCTTCACTAACCTCCTACCTTGA-
CCCTCTCTTTTAGGGCTCTTTCTGACCTCCACCATGA-
TACTAGGACCCCATGTTCTGTACCCTCTTGACTC-
TATGACCCCCACTGCCACTGCATCCAGCTGGGTCCC-
CTCCTATCTCTATTTCCAGCTGGCCAGTGCAGTCTCA-
GTGCCCCACTGTTTGTGCTAAGTCTGAAGGGCTG-
ACATTTTACTGACTTGCAAACAAATAAGCTAAGTTTC-
CAGAGTTTTGTGAATGCTGGCAGAGTCCATGAGACT-
CCTGAGTCAGAGGCAAAGGCTTTTACTGCTCACAGC-
TTAGCAGACAGCATGAGGTTTCATGTTACATTAGTAC-
ACCTTGCCCCCCCCAAATCTTGTAGGGTGACCAGAGC-
AGTCTAGGTGGATGCTGTGCA.

Real-Time RT-PCR Primers.

KLK3 forward, GATGAAACAGGCTGTGCCG;
KLK3 reverse, CTCACAGCTACCCACTGCA;
KLK3e S1 forward, TGGGACAACCTTGCAAACCTG;
KLK3e S1 reverse, CCAGAGTAGGTCTGTTTTCAATCCA;
KLK3e S2 forward, AGAATTGCCTCCCAACACTG;

KLK3e S2 reverse, GGACCCACTCCTCACTCAAA;
KLK3e S3 forward, GATGTGGGAATGGCCTAAGA;
KLK3e S3 reverse, GGATTAGGTGGCAAGATGGA;
KLK3e S4 forward, AGGGTATCACCAGCCCTTCT;
KLK3e S4 reverse, GAGGATGTCGGCAGCTCTAC;
KLK3e S5 forward, GCCAGCATCAGCCTTATCTC;
KLK3e S5 reverse, TACAACCACATCCCCGTTCT;
KLK3e S6 forward, TTGTGCTTCGGATGAGTTTG;
KLK3e S6 reverse, CCATTGTTTGATTCCCCATC;
KLK3e S7 forward, GGTCAAGTTTTTGGTTGAGGA;
KLK3e S7 reverse, TGAGGGAGACAGAGGTGGAT;
KLK3e AS4 forward, TCCGGAACACAGATCTGACA;
KLK3e AS4 reverse, GAACGGATCACGGGTAAAGA;
KLK3e AS3 forward, AAATCTGGCAAGGGAGAGGT;
KLK3e AS3 reverse, GAGTTCCCTGGATGGCAATA;
KLK3e AS2 forward, GACGATCAAATGTGGTCACG;
KLK3e AS2 reverse, CTCCATCAAATGAGGCCAGT;
KLK3e AS1 forward, TTTTGTGAATGCTGGCAGAG;
KLK3e AS1 reverse, TGCACAGCATCCACCTAGAC;
KLK2 forward, GCTGCCCATTCCTAAAGAAG;
KLK2 reverse, TGGGAAGCTGTGGCTGACA;
KLK15 forward, TAACTGTGGCGCTTCCCTCATC;
KLK15 reverse, AGACGTGGTCCGTAGTTGCTCT;
KLK4 forward, GGCACTGGTCATGGAAAACGA;
KLK4 reverse, TCAAGACTGTGCAGGCCAGCC;
NKX31 forward, CGCAGAACGACCAGCTGAGCA;
NKX31 reverse, CCTGAAGTGTTTTAGAGTCCAAC;
NKX31e forward, GCCCAGTTCATTCTTTTGAA;
NKX31e reverse, CCAAGTCAAAGGGGAATCCT;
TMPRSS2 forward, CCTGTGTGCCAAGACGACTG;
TMPRSS2 reverse, TTATAGCCCATGTCCCTGCAG;
FKBP5 forward, TGCCAAGACCTTCCGAGGCGTC;
FKBP5 reverse, GGCTTCCGCTCTGCCCAAAGA;
FKBP5e forward, GCGGTGTCTTTTCGTAGAGG;
FKBP5e reverse, TAAGGCCCTTCTCTATTCT;
PLZF forward, AGCGGGTACTGCGAACTGC;
PLZF reverse, ATCGCACAAAGTCCCGGCCAG;
PLZFe forward, AGACCAGGCCAAAATGTCAG;
PLZFe reverse, TTTGTTTCCCCTCCTCTCT;
PDE9A Forward, GATCCCAATGTTTGAAACAGTGAC;
PDE9A reverse, TCCCAAAGTGGCTGCAGC;
GUCY1A3 forward, AGCAGTGTGGAGAGCTGGAT;
GUCY1A3 reverse, CTGATCCAGAGTGCAGTCCA;
GAPDH forward, CCTGTTTCGACAGTCAGCCGCATC;
GAPDH reverse, GGTGACCAGGCGCCCAATACG.

PCR Primers for Constructing Plasmids.

KLK2 promoter luc forward, GATCGAGCTCGAGTTCAA-
GGCTACAGGGAGC;
KLK2 promoter luc reverse, GATCCTCGAGGGGCTTCA-
GCATAAGAGTCAG;

KLK2 promoter (−407 ~+143) was subcloned into pGL3 basic
using SacI and XhoI.

KLK3e S1_promoter luc, GATCGGATCCGAATTCTGGG-
ACAACCTTGCAAACCTGC;
KLK3e S7_promoter luc, GATCGTCGACTGAGGGAGA-
CAGAGGTGGATA;
KLK3e S7_promoter luc, GATCGTCGACTGGGACAAC-
TGCAAACCTGC;

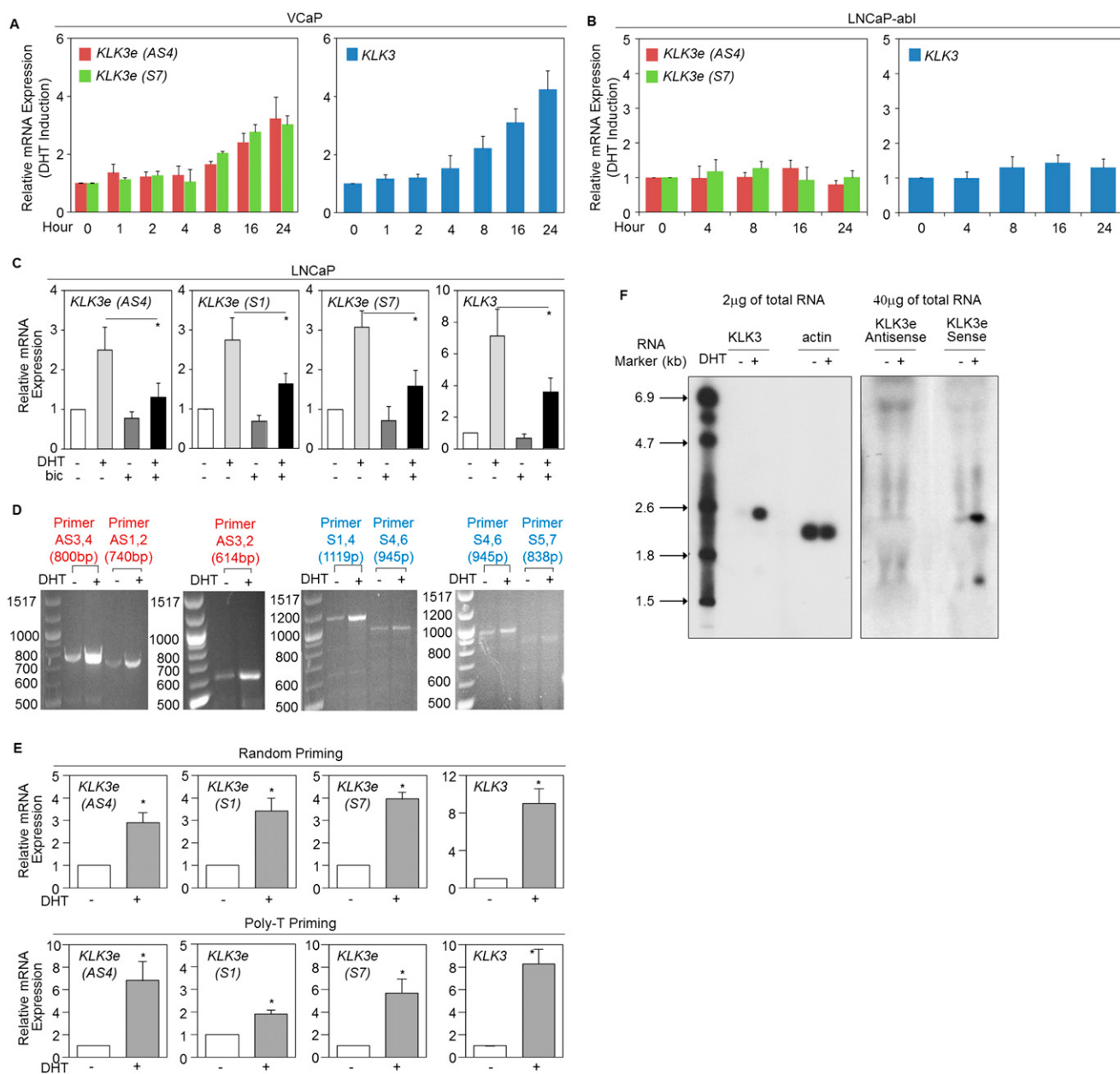
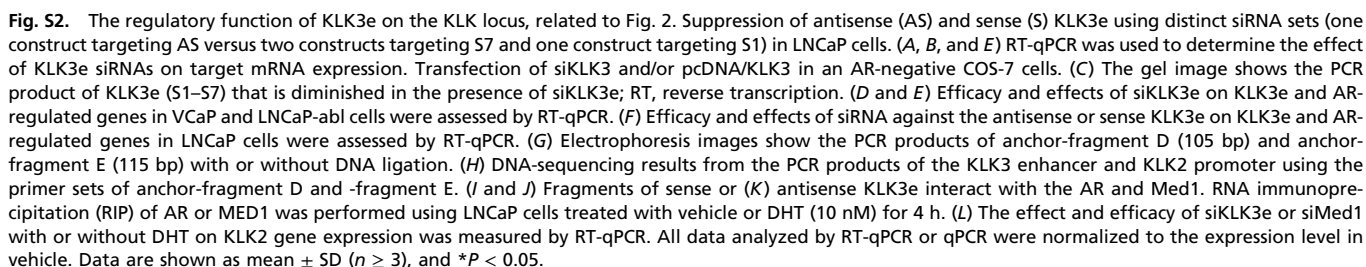


Fig. S1. The KLK3 enhancer produces long noncoding RNAs, related to Fig. 1. (A and B) VCaP and LNCaP-abl cells were treated with DHT (10 nM) in a time-dependent manner. The expression of antisense (red bar), sense (green bar), or mature KLK3 (blue bar) was determined by reverse transcription–quantitative PCR (RT–qPCR). (C) LNCaP cells were treated with or without 10 nM DHT and 10 μM bicalutamide (bic). The expression of KLK3e and KLK3 was measured by RT–qPCR. (D) Electrophoresis images show that the PCR products from fragments of antisense and sense KLK3e upon DHT (10 nM) stimulation for 24 h. (E) Reverse transcription was performed using random hexamer primers and 3'-RACE (poly-T) adapter from the same RNA materials isolated from LNCaP cells treated with or without DHT (10 nM). The expression of KLK3e and KLK3 was measured by RT–qPCR. (F) RNA was isolated and purified from LNCaP cells treated without (–) or with (+) DHT. Two or 40 μg of total RNA was subjected to electrophoresis, followed by hybridization using the LNA probes against KLK3, actin, and antisense and sense KLK3e whose targeting regions are shown in Fig. 1C. All data analyzed by RT–qPCR were normalized to individual expression in the absence of DHT exposure. Data represent mean ± SD ($n \geq 3$), and $*P < 0.05$.



A

KLK2

Relative mRNA Expression

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

KLK2

Relative mRNA Expression

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

Med1

Relative mRNA Expression

Veh - + - + - +
DHT (2hr) DHT (8hr)

B

KLK3 Promoter

ChIP:AR

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:PolII(S5p)

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:IgG

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

KLK3

Relative mRNA Expression

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

C

KLK3 Promoter

ChIP:AR

% Input

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:PolII(S5p)

% Input

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:IgG

% Input

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

KLK3

Relative mRNA Expression

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

D

KLK3 Promoter

ChIP:Med1

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:IgG

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

KLK3

Relative mRNA Expression

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

E

KLK3 Enhancer

ChIP:AR

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:PolII(S5p)

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:IgG

% Input

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

KLK3e

Relative mRNA Expression

siKLK3e - + - + - +
Veh DHT (2hr) DHT (8hr)

F

KLK3 Enhancer

ChIP:AR

% Input

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:PolII(S5p)

% Input

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

ChIP:IgG

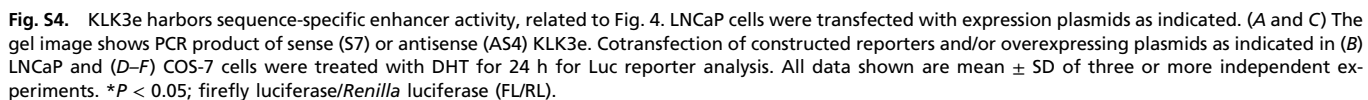
% Input

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)

KLK3e

Relative mRNA Expression

siMed1 - + - + - +
Veh DHT (2hr) DHT (8hr)



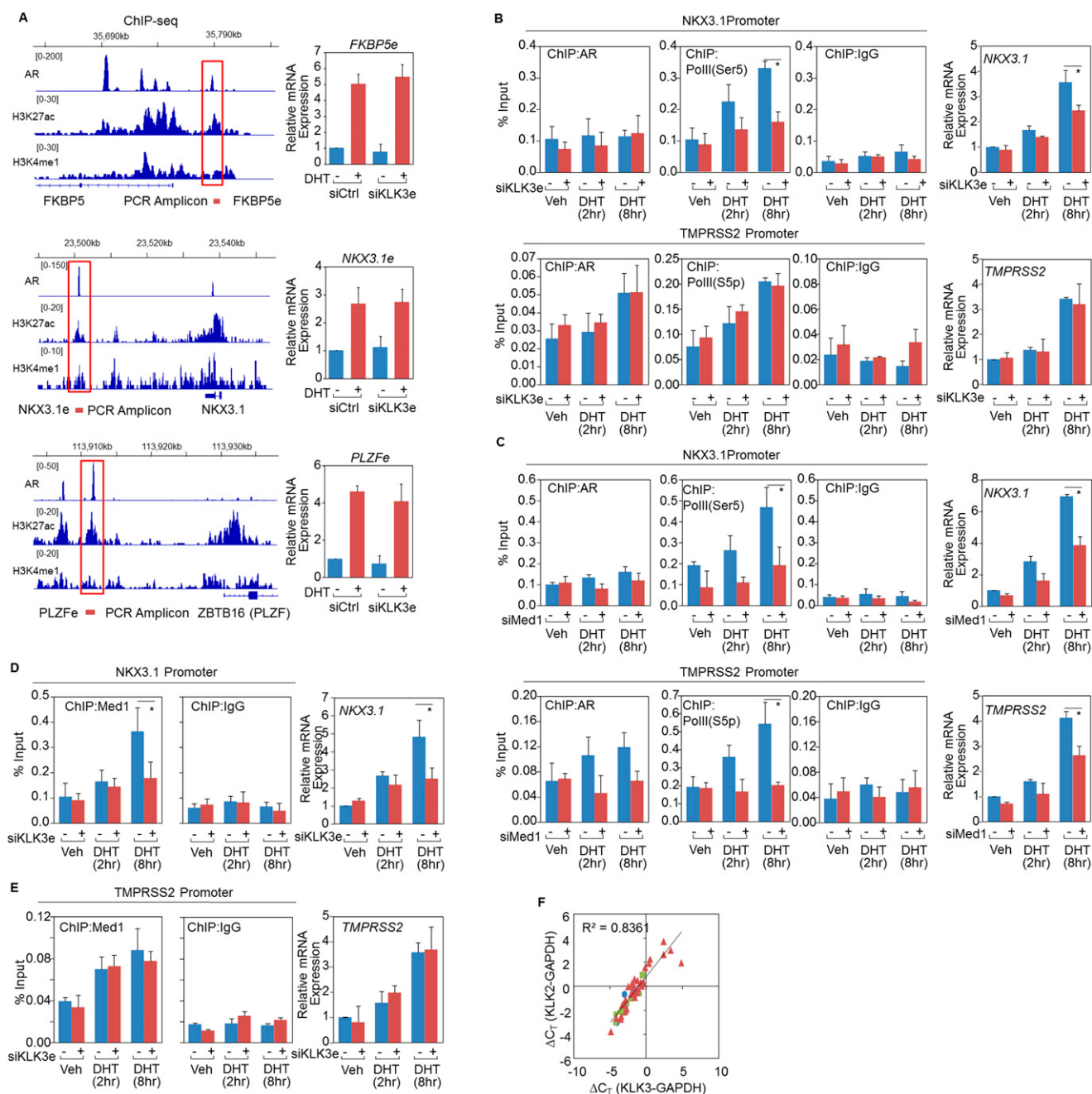


Fig. S5. KLK3e selectively regulates the promoter activity of target gene, related to Fig. 5. (A) The chromatin status at the upstream enhancers of FKBP5, NKX3.1, and PLZF. ChIP-seq datasets of AR, H3K27ac, and H3K4me1 (1). RT-qPCR analyses of the expression of eRNAs with or without KLK3e silencing and/or DHT (10 nM) treatment in LNCaP cells. ChIP of AR or Med1 or activated Pol II (S5p) was performed using LNCaP cells transfected with (B, D, and E) siKLK3e or (C) siMed and treated with or without DHT (10 nM) for 2 or 8 h. qPCR analysis was used to assess the promoter activity of NKX3.1 and TMPRSS2. mRNA expression levels of NKX3.1 versus TMPRSS2 in KLK3e- and Med1-depleted LNCaP cells were determined by RT-qPCR. (F) Correlation between KLK3 and KLK2 gene expression in vivo. All data were normalized for GAPDH by the differences in threshold cycles (ΔC_T) from the normal human prostate gland ($n = 4$), primary ($n = 11$) and metastatic ($n = 32$) prostate tumors (2). All data shown are mean $\pm$ SD of three or more independent experiments, and $*P < 0.05$.

1. Yu J, et al. (2010) An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. *Cancer Cell* 17(5):443–454.
2. Stanbrough M, et al. (2006) Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res* 66(5): 2815–2825.