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The expression of glucocorticoid receptor is negatively regulated by active androgen receptor signaling in prostate tumors

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The glucocorticoid and androgen receptors (GR and AR) can commonly regulate up to 50% of their target genes in prostate cancer (PCa) cells. GR expression is stimulated by castration therapy, which has been proposed to be one mechanism that compensates for AR signaling blockade and promotes castration-resistant PCa (CRPC) progression. However, whether GR functions as a driver for CRPC or a marker reflecting AR activity remains unclear. Here, we applied PCa tissue microarrays to show that GR protein levels were elevated by castration therapy, but reduced to pre-castration levels when tumors were at the CRPC stage. Using subrenal capsule xenograft models, we showed that GR expression was inversely correlated with AR and PSA expressions. GR expression levels are not associated with tumor invasion and metastasis phenotypes. In castration-resistant C4-2 xenografts expressing AR shRNA, regressing tumors induced by AR knockdown expressed higher levels of GR and lower levels of PSA than non-regressing tumors. Immunoblotting and real-time PCR assays further showed that AR knockdown or AR antagonists increased GR expression at both mRNA and protein levels. ChIP combined with DNA sequencing techniques identified a negative androgen responsive element (nARE) 160K base pairs upstream of the GR gene. Gel shift assays confirmed that AR directly interacted with the nARE and luciferase assays demonstrated that the nARE could mediate transcription repression by ligand-activated AR. In conclusion, GR expression is negatively regulated by AR signaling and may serve as a marker for AR signaling in prostate tumors.

The primary treatment for metastatic prostate cancers (PCa) is androgen deprivation therapy (ADT). Although initially responsive, tumors eventually progress into the incurable stage referred to as the castration-resistant prostate cancer (CRPC).[1] Many mechanistic studies have demonstrated that androgen receptor (AR) signaling plays a critical role during CRPC progression.[2,3] Knocking down AR expression by RNAi or blocking AR activity by antagonists sufficiently reduced PCa tumor growth.[4–6] However, even with the most stringent inhibition of the AR signaling many tumors still recur[7] and no curative treatments are currently available. Global gene profiling studies identified many genes whose expressions were dramatically upregulated by androgen deprivation conditions or by AR RNA silencing.[4,8] Some of these genes involve in critical survival pathways that are adapted by cancer cells to counteract ADT.[8] These genes are ideal targets for anti-PCa drug development. Many other genes (*e.g.*, PSA) do not play significant roles for cancer cell survival, but could be used as biomarkers to monitor the activity of AR and tumor progression.

Amino acid sequence of the glucocorticoid receptor (GR) is highly homologous to the AR in the DNA binding domains.[9] GR and AR share approximately 26–46% overlap of their cistromes pending upon cell contexts[10] and over 50% of targeted genes can be commonly regulated by both receptors.[11] Importantly, androgen deprivation conditions induce GR expression, which change has been proposed to be one of the mechanisms that is utilized by the tumors to compromise the repressive impacts by ADT.[11,12] However, CRPC patients post chemo- and radiation-therapy are treated commonly with GR agonists due to their actions of antiemeric, anti-inflammatory, pain relieving and anti-androgen synthesis.[13] Yet, no adverse impacts on tumor progression have been observed.[14–16] These observations raise the controversial roles for GR signaling in CRPC tumors and further investigations are warranted.

Key words: androgen receptor, glucocorticoid receptor, prostate tumor
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What's new?

Many prostate cancers (PCa) eventually develop resistance to androgen-deprivation therapy, and the androgen receptor (AR) is known to play a critical role in this process. However, even when AR signaling is blocked, PCa tumors may still recur. Some studies have suggested that the glucocorticoid receptor (GR) might be responsible for the progression to castration-resistant prostate cancer. In this study, however, the authors determined that this is not the case. They also found that GR expression is suppressed by active AR signaling, due to a "negative androgen-response element" sequence near the GR gene.

Upregulation of GR expression by androgen depleted conditions also suggested that GR may be a biomarker reflecting the activity of AR signaling. The AR was reported to bind its targeted genes, including the AR gene itself, to suppress these gene transcriptions.[17,18] It still needs to determine whether the similar mechanism is also applied to GR transcription suppressed by the AR. In this study, we measure the GR expression in prostate tumors and define a molecular mechanism by which AR represses GR gene transcription through recognizing a nARE in the GR gene promoter.

Materials and Methods

Human tissue microarray and immunohistochemistry (IHC)

Information on human prostate tissue microarray (TMA) was published.[19,20] IHC was performed by Ventana Discovery XT autostainer (Ventana) with GR and AR antibodies as reported.[21] Stained slides were scanned by a Leica SCN400 scanner. Digital images were evaluated and scored by pathologist (L.F). The percentage of stained cells (0–16%, 17–33%, 34–66% and 67–100%) and the staining intensity (no staining, low, moderate and high intensity staining) as 0–3 were recorded. The IHC scores were calculated by the index of HSCORE=$\Sigma pi(i+1)$, where i = the intensity of staining and pi = the percentage of stained cells.

Tumor xenografts

Subrenal capsule (SRC) xenografts are human prostate tumor tissue trunks that were grafted underneath renal capsules of SCID mice as reported previously.[22–24] Animals were sacrificed for necropsy three to six months post implantation pending upon tumor doubling time of each SRC model and on the health status of the hosts. Xenografts were harvested and fixed for IHC analyses. The hosts were examined for metastases of human origin in lymph nodes, lungs, livers, kidneys, spleens and bones (femur). Castration-resistant C4-2 xenografts expressing AR shRNA was also reported previously.[4,5] Briefly, C4-2 cells containing inducible AR shRNAs were inoculated into castrated NUDE mice ($n = 16$). When the tumors became palpable, tumor volumes and serum PSA were measured weekly. When PSA levels reached 50–75 ng/mL, the mice were given 200 ng/mL of doxytetracycline (Dox) (Sigma Chemicals). Three weeks post Dox administration, mice which showed decreases in tumor volumes and PSA concentrations were classified as regressors ($n = 8$), while mice which did not respond were classified as non-regressors ($n = 8$). Three animals from each group were euthanized and their tumors were removed. Tumor tissue samples were used to extract RNA for real-time qPCR. They were also fixed and paraffin embedded for IHC assays. Dox treatment was continued to the remaining animals. When the tumor volumes exceeded 20% of the body weight or when they had completely regressed, the mice were euthanized and the tumors were collected. All animal procedures were performed according to the guidelines provided by the Canadian Council of Animal Care and with institutional certification.

Real-time qPCR, western blotting, chromatin immunoprecipitation (ChIP) and ChIP sequencing

Real-time PCR assays were performed to validate the findings following the protocol reported previously.[25] Immunoblotting, ChIP and ChIP-seq assays were performed as reported.[26,27] Antibody information and DNA sequences obtained from ChIP-seq were listed in supplementary materials.

Gel shift assay

Gel shift assays followed the protocol described previously with minor modifications.[28] Biotin labelled DNA oligos (IDT Inc.) were used as primers to amplify a 75 base pair (bp) DNA fragment covering the nARE or the nARE(m) (see Supporting Information). DNA fragments were purified by QIAquick PCR purification kit (Qiagen) and 2ng purified DNA were used in Gel Shift assay. AR-DBD were purified by Glutathione Separose 4B (GE Healthcare) as reported.[29] AR-DBD and DNA probe were incubated in buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 10% glycerol (v/v) plus 1 mM DTT) together with 2 mg/mL poly(dI-dC) in a final volume of 25 μL. Reactions were incubated in room temperature for 20 min before loaded onto 5% acrylamide/bis gel (29 : 1) in the presence of 0.5xTBE running buffer. DNA/protein complex were then transferred to Biodyne B pre-Cut Modified Nylon Membranes 0.45 μm (Thermo Scientific) at 25V for 40 min. The shifted DNA bands were detected by Chemiluminescent Nucleic Acid Detection kit (Thermo Scientific).

Luciferase reporter assay

Genomic DNA fragment containing the nARE from GR promoter at the chromosome 5 were amplified by PCR with primers listed in supplementary material. DNA fragments were cloned into pGL3 promoter luciferase vector. Site mutagenesis was performed to at the nARE site using Q5 Site-Directed

Mutagenesis Kit (NEB). Luciferase reporter assays were performed as previous reports.[30]

Statistics

Results are expressed as the mean ± SEM. To determine differences between groups, Mann-Whitney or student *t*-test was carried out using GraphPad Prism (version 4) with the level of significance set at $p < 0.05$ as *, $p < 0.01$ as ** and $p < 0.001$ as ***.

Results

GR protein expression is elevated by neoadjuvant hormone therapy (NHT) in human prostate tumors

To investigate GR expression during prostate cancer progression, we applied IHC on human PCa tissue microarrays.[19,20] IHC signal for GR was localized in the nuclei of prostate epithelial cells as well as a sub-group of stromal cells. Particularly, vascular endothelial cells showed strong nuclear expression (Fig. 1). Pathological scoring of GR in luminal epithelial cancer cells showed that GR protein levels increased 50% in the tumor group after 1–5 month NHT treatment ($p = 0.04$) and 100% in tumor groups after 6–8 ($p = 0.0007$) and 9–12 month ($p = 0.0014$) NHT treatment (Fig. 1*a*). However, when tumor progressed into the CRPC stage, GR protein levels dropped to the pre-NHT treatment levels ($p = 0.4$). Representative IHC images of GR staining on NHT-treated tumor tissues are shown in Figure 1*b*. These results indicated that blocking AR signaling increased GR expression in human prostate tumors. Since re-activation of AR is generally believed to contribute to CRPC progression in many tumors, reduced GR expression was observed in CRPC tumors further supported that reactivated AR signaling repressed GR expression. We further showed that GR protein expressions were at relatively similar levels in tumors with various Gleason scores (Fig. 1*c*). Within the CRPC tumor group, all tumors ($n = 21$) had strong AR expression, which was consistent with overall lower levels of GR protein. Eight tumors were PSA positive but GR negative, two tumors were PSA negative but GR positive and 11 tumors are both PSA and GR positive (Fig. 1*d*). These results indicated that PSA expression, the functional index of AR activation, was inversely correlated with GR positivity in ∼50% of CRPC tumors. Even though the other 50% tumors showed both GR and PSA positive, the pathological scores of GR in these tumors were significantly lower than NHT-treated tumor groups (Fig. 1*a*).

GR expression is suppressed by AR signaling in human PCa xenografts

We have also measured GR, AR and PSA protein expression in several SRC grafts derived from either hormone naïve or CRPC tumors (Fig. 2*a*). All hormone naïve tumors expressed strong AR and PSA proteins, but low or negative GR expression. In CRPC tumor models, LTL412 grafts expressed AR and PSA but not GR protein. By contrast, both LTL370 and LTL545 were AR and PSA negative, but expressed high levels of GR protein. These results were consistent to our findings in human tumor biopsies that GR expression was inversely correlated with AR activity.

Metastasis and local invasion properties of SRC tumor models[12] were also aligned with GR expression in Figure 2*a*. Representative H&E images of tumors and host kidneys were presented in Figure 2*b* and Supporting Information Figure S1. Both 313B and 412 tumors, regardless of their GR expression levels, showed very limited local invasion as indicated by a clear boundary between tumors and host kidneys. Consistent with their poor local invasiveness, these tumors did not show metastasis in distal organs (Fig. 2*a*). Although both 370 and 545 tumors expressed high levels of GR, only 370 tumors showed apparent local invasion (indicated by the deep penetration of tumor cells into renal parenchyma) and distal metastasis. In addition, 313A, 313D and 313B, all derived from the same cancer patient, showed different invasive and metastatic ability, which were also not correlated with GR expression.

Another CRPC tumor model was the C4-2 xenografts expressing AR shRNA that were grafted in castrated NUDE mice. These tumors presented bipartite tumor growth with ∼50% of the tumors presenting a continuous regression pattern, while the other 50% tumors showing a non-regressive phenotype even under castration conditions in addition to AR knockdown (Fig. 3*a*). Serum PSA levels also showed separated trends. Real-time qPCR further confirmed that there were 50% decreases of PSA mRNA levels, but 7-fold increases of GR mRNA levels in non-regressing tumors compared with regressing tumors (Fig. 3*b*). AR and GR expressions were also detected by IHC on back-to-back tumor slides from regressing and non-regressing tumors (Fig. 3*c*). Although AR protein is significantly reduced in both tumor groups, portions of tumor cells sustained low levels of AR protein. While many cells are both AR and GR negative, interestingly though cells expressing GR are all AR negative. Together, these findings further supported that GR expression was repressed by active AR signaling. Higher GR expression was expressed in regressing C4-2 xenografts.

AR suppressed GR expression in prostate cancer cell lines

Using immunoblotting assays, we further showed that GR was widely expressed in prostate cancer cell lines (Fig. 4*a*), including LNCaP derived castration-resistant LN(AI) and LN95 cells. In AR negative PC3 cells, introduction of exogenous AR protein reduced GR protein levels (Fig. 4*b*). In AR positive LNCaP cells, AR knockdown or androgen-depleted culture medium induced GR protein levels (Fig. 4*c*). Consistently, GR expression was inversely correlated with PSA secretions, a marker reflecting AR activation. In addition, we found that the AR agonist, DHT, inhibited GR mRNA levels, while AR antagonists or androgen-depleted serum induced GR mRNA levels in LNCaP and

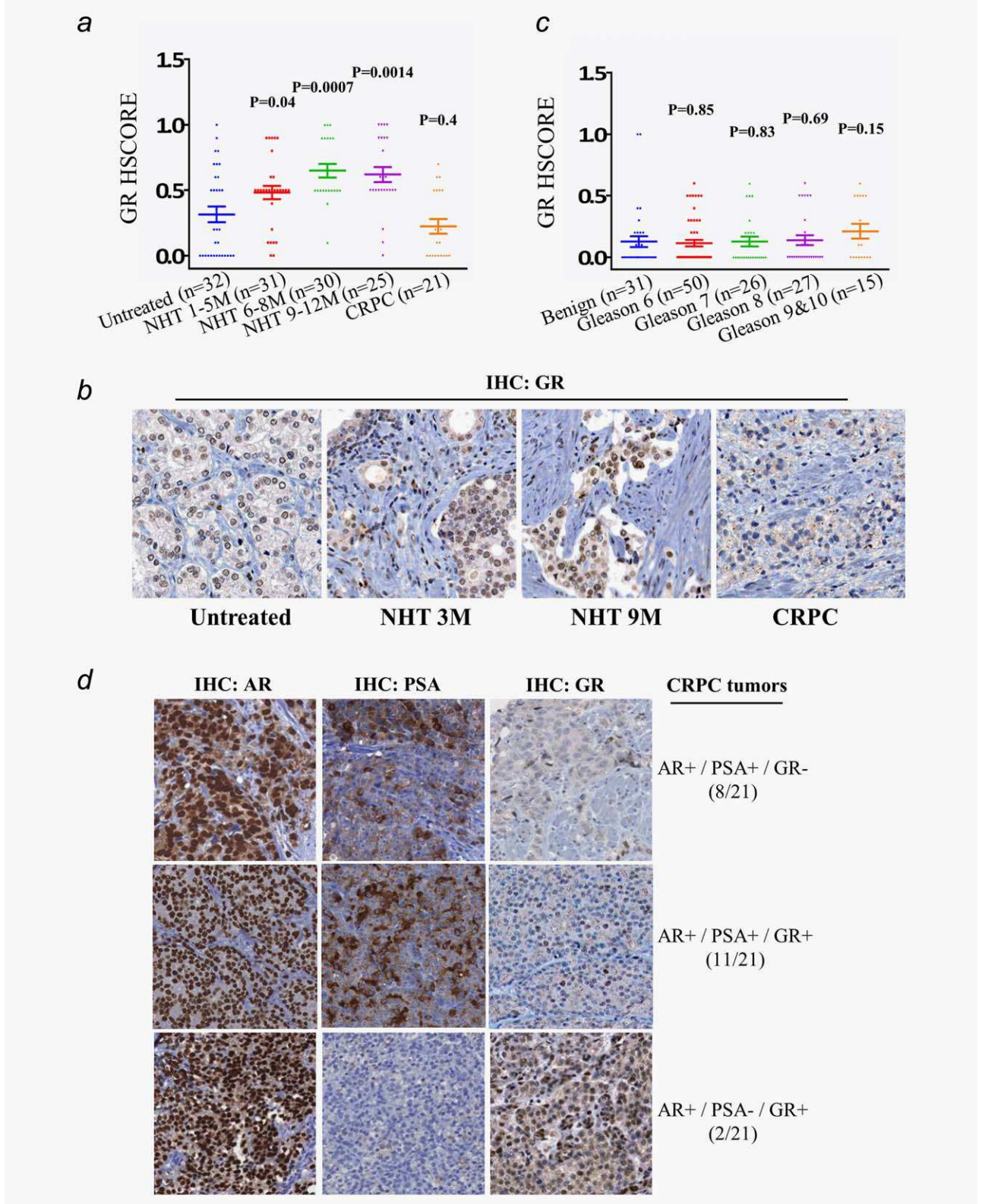

Figure 1. GR protein expression is induced by castration therapy in prostate tumor biopsies. (*a*) Human prostate cancer tissue microarray containing untreated, NHT-treated, and CRPC patient tumor samples were immunostained with GR antibody. HSCOREs of epithelial cells were analyzed as described in the Materials and Methods sections. Mann-Whitney tests compared between untreated group and NHT or CRPC group and between CRPC and NHT groups. (*b*) Representative IHC images from untreated, NHT treated and CRPC tumor samples were presented. (*c*) Benign or prostate cancer samples with different Gleason scores were immunostained with GR antibody. Mann-Whitney test compared the benign group with each neoplastic group. (*d*) Representative immunostaining of AR, PSA, and GR protein expressions in the CRPC tumor group.

a

Tumor Line ID	Original Tumor	Androgen Sensitivity	AR	PSA	GR	Metastasis	Local Invasion
LTL418	Hormone Naïve	+	+++	+++	-	-	-
LTL313A	Hormone Naïve	+	+++	+++	-/+	+	+
LTL313B	Hormone Naïve	+	+++	+++	+	-	-
LTL313D	Hormone Naïve	+	+++	+++	-	+	+
LTL313H	Hormone Naïve	+	+++	+++	+	+	+
LTL331	Hormone Naïve	+	+++	+++	+	+	+
LTL467	Hormone Naïve	+	+++	+++	-/+	+	+
LTL412	CRPC	+	+++	+++	-	-	-
LTL370	CRPC	-	-	-	+++	+	+
LTL545	CRPC	-	-	-	+++	-	-
LTL313BR	CRPC	+	+++	+++	-/+	ND	+
LTL418R	CRPC	+	+++	+++	-/+	ND	+

b

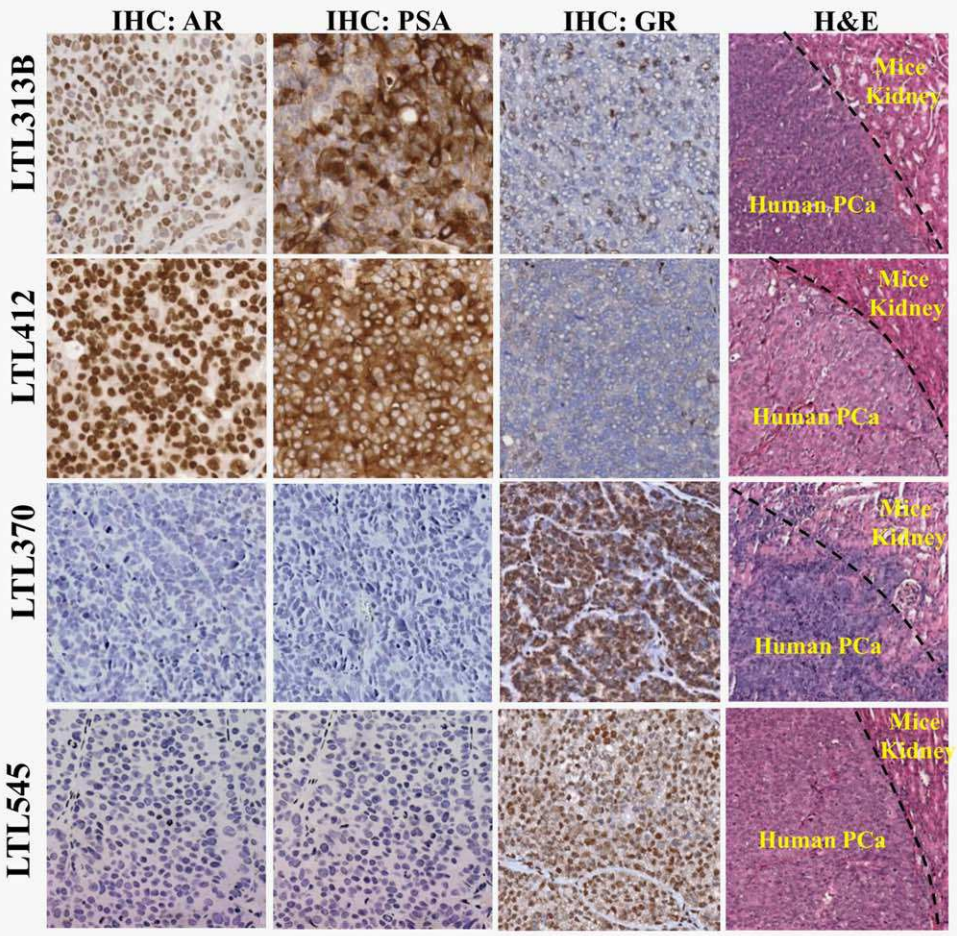

Figure 2. Inverse correlation of GR expression with AR and PSA expressions in SRC tumor models. (*a*) Seven SRC tumor models with three tumors per model were immunostained with AR, PSA, and GR. Information on each SRC model including their invasion phenotypes were listed. (*b*) Representative IHC images of LTL313B, LTL412, LTL370, and LTL545 tumors stained with AR, PSA, and GR antibodies were presented. H&E staining at the boundary of tumors and host kidneys are also shown.

LNCaP derived cell lines (Fig. 4*d*). Knockdown of AR protein by shRNA alleviated the suppressive effects of DHT (Fig. 4*e*). Together, these results demonstrate that ligand-activated AR inhibited GR expression at both mRNA and protein levels, suggesting that AR is a negative regulator of GR transcription in PCa cell lines.

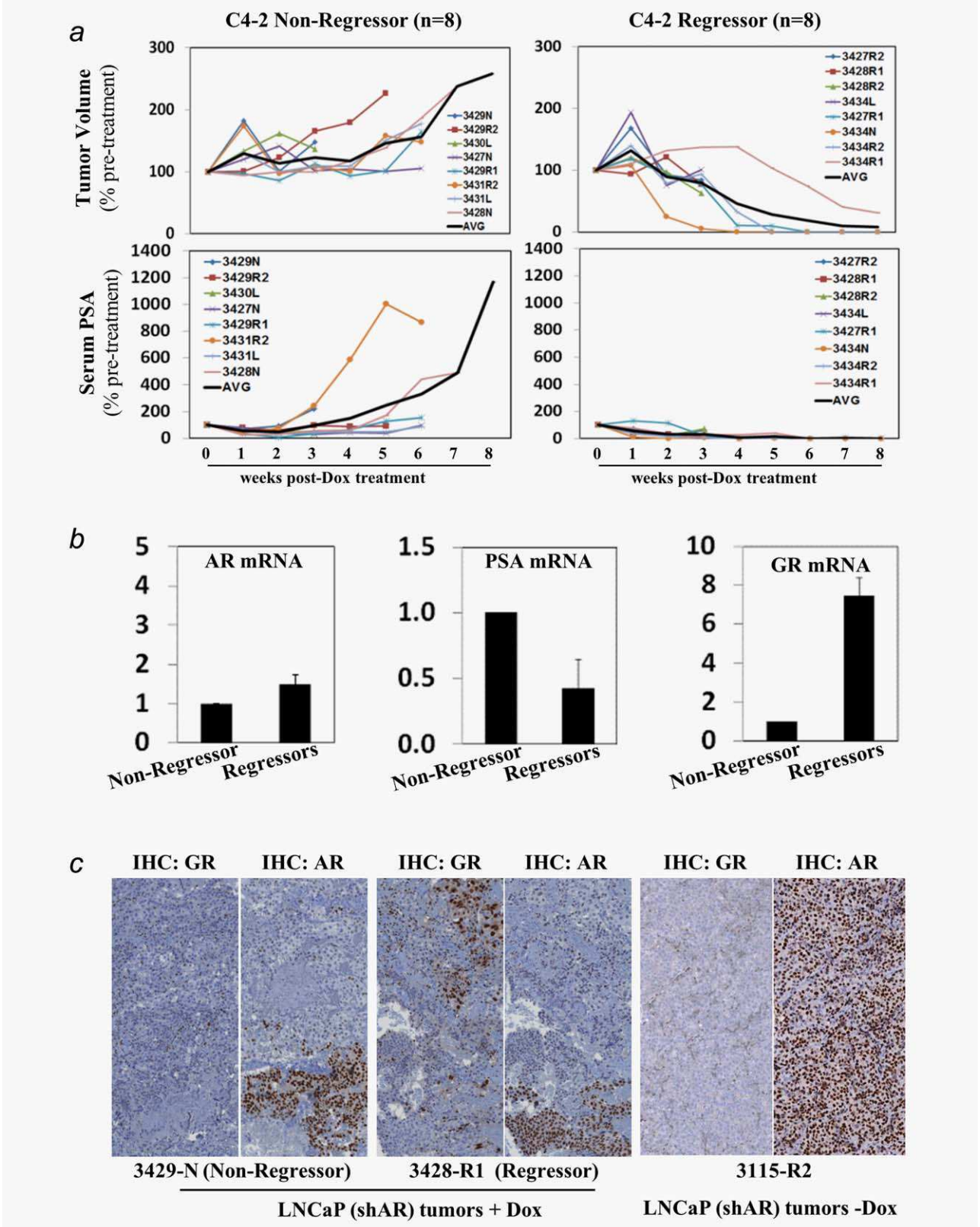

Figure 3. AR and GR expressions in castration-resistant C4-2 xenografts. (*a*) Tumor volumes and serum PSA concentrations were measured in 16 nude mice bearing castration-resistant C4-2 xenografts with inducible AR shRNA. Half of the tumors were defined as regressors and the other half defined as non-regressors as described in the Materials and Methods section. Black lines indicate the trend changes of all 8 animals. (*b*) Real-time PCR measured AR, PSA, and GR mRNA levels from nonregressors ($n = 3$) and regressors ($n = 3$) 3 weeks post Dox treatment. (*c*) Back-to-back tissue slides from C4-2 xenografts were stained with AR and GR antibodies. Representative images were presented from 6 tumors.

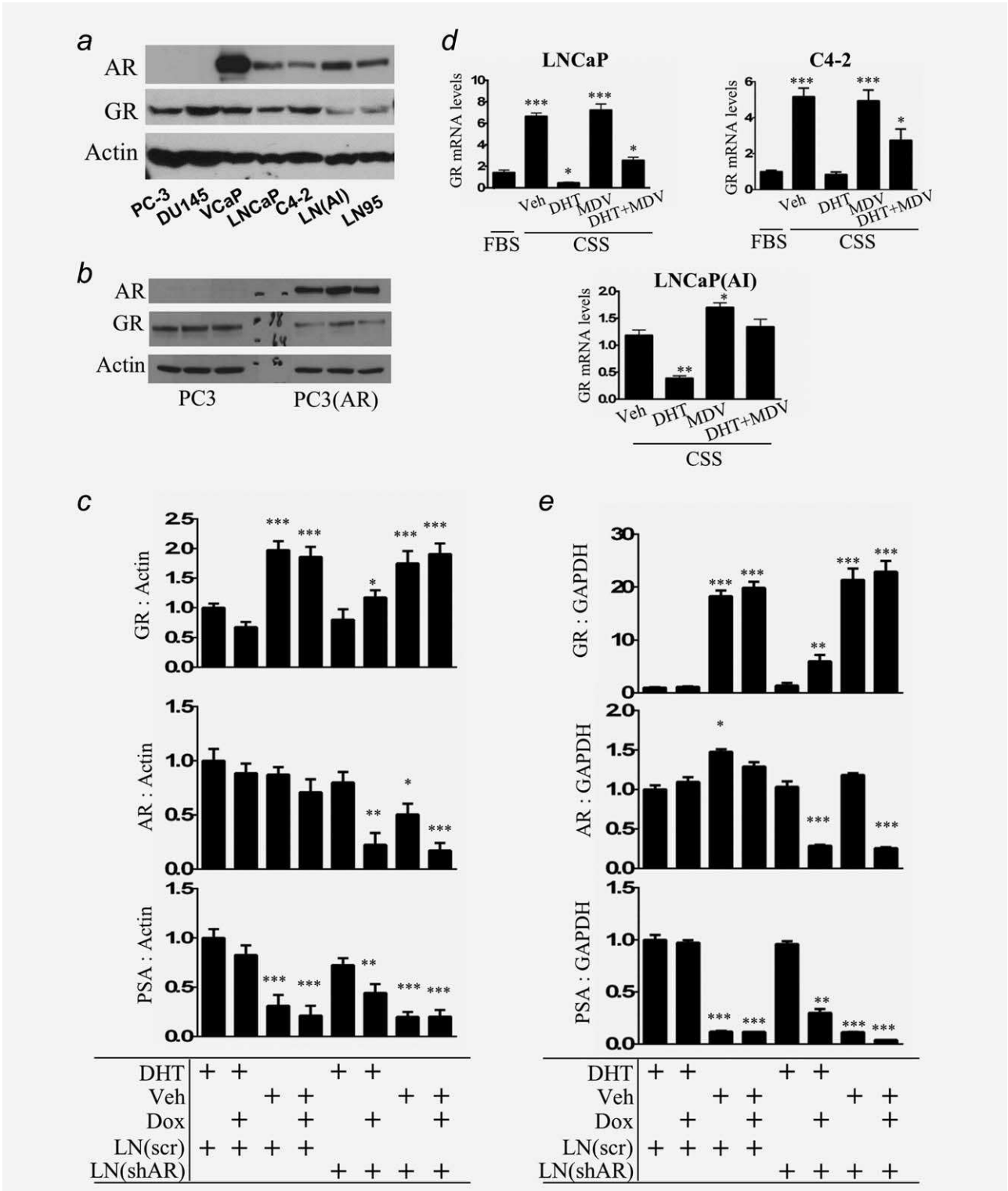

Figure 4. AR negatively regulates GR protein levels in prostate cancer cells. (*a*) Protein lysates were collected from seven prostate cancer cell lines. (*b*) Protein lysates from PC-3 and PC-3(AR) cells were extracted from triplicate experiments. Immunoblotting assays were performed with AR, GR, and beta-Actin antibodies. (*c*) LNCaP cells carrying inducible control or AR shRNA were maintained in androgen-depleted medium for 48 hr. Cell were then treated with ±2 μg/ml Dox for 48 hr and ±10 nM DHT for another 24 hr. Immunoblotting assays were performed with AR, GR, PSA, and beta-Actin antibodies. Densitometry of protein bands were calculated by Image J software. Relative protein levels of AR, GR, and PSA protein levels were calibrated Actin and plotted from three independent experiments. Representative western blot images were shown in supplementary figures. (*d*) LNCaP, C4-2, and LN(AI) cells were maintained in androgen-depleted medium and then treated with vehicle, 10 nM DHT, and/or 25 μM MDV3100 for 16 hr. (*e*) LNCaP cells carrying inducible AR shRNA were treated with ±2 μg/ml Dox for 48 hr and ±10 nM DHT for 16 hr. Relative mRNA levels of GR, AR, and PSA to GAPDH were measured by real-time qPCR. Data are presented as mean ± SEM from three independent experiments. Student's *t*-test compared data between treatment groups and control groups on the first bar of each figure with $*p < 0.05$; $**p < 0.01$, and $***p < 0.001$.

Identification of an androgen response element in the GR gene promoter

To identify the potential binding sites of AR on the GR gene, we performed ChIP-seq assays.[27] LNCaP and C4-2 cells were cultured in charcoal stripped serum and treated with either vehicle or DHT for 4 hr. AR antibody precipitated a 452 bp DNA fragment at ~160K bp upstream of the transcription initiation site of the GR gene (Fig. 5*a*). This was the only AR binding site at or near the GR gene that could be identified. A consensus ARE sequence was located and termed as nARE. ChIP assays further confirmed that AR recruitment to the nARE was DHT-dependent (Fig. 5*b*). These results were replicated in VCaP and LN95 PCa cell lines. A region near the nARE and the region containing PSA enhancer were used as negative and positive controls respectively.

To determine whether AR directly interacted with the nARE, we performed gel shift assays. Biotin-labeled DNA fragments containing the nARE or the mutant nARE were incubated with purified AR-DBD protein (Fig. 5*c*). AR induced up-shifted DNA bands containing the nARE, but not the mutant nARE, in a protein dose-dependent manner. In addition, non-labeled nARE, but not the mutant nARE oligos competed AR protein from interacting with the nARE (Fig. 5*d*). AR protein samples used in the gel shift assays were coomassie stained as shown (Fig. 5*e*).

The nARE regulates transcription suppression of the GR gene by AR

To confirm that the nARE can mediate AR suppressive effects on GR transcription, we cloned the GR promoter region containing the nARE into a luciferase reporter vector, termed as 160k-Luc (Fig. 6*a*). The luciferase vector carrying mutant nARE was referred to as 160k(m)-Luc and luciferase vector carrying a 1.5k bp fragment of the GR gene around the transcription initiation site was designated as 1.5k-Luc. Transfection of the 160k-Luc reporter with the AR expression plasmid into LNCaP cells resulted in dose-dependent suppression of luciferase activity (Fig. 6*b*). Additionally, increasing doses of DHT strengthened the suppressive effects of endogenous AR on the luciferase activity of the 160k-Luc reporter (Fig. 6*c*). AR mediated repression was dependent upon the presence of the nARE, as both 160k(m)-Luc and 1.5k-Luc did not respond to DHT treatment (Fig. 6*d*). These results demonstrated that the nARE was functional and can mediate suppression of downstream gene transcription.

Reduced histone acetylation often accompanies gene transcription silencing. To study whether the recruitment of AR to the nARE was associated with decreased histone deacetylation, we performed ChIP assays. While total histone 3 levels remained at similar levels, there were ~50% decreases in acetylated-histone 3 levels at the nARE region as well as the regions at exon 1 and exon 2 of the GR gene following DHT administration (Fig. 6*e*). On the contrary, acetyl-histone 3 level at the control region next to the nARE did not change with DHT treatment. These results suggested that the nARE can recruit ligand-activated AR to mediate a long-range regulation of GR gene transcription.

DISCUSSION

This study reports on several novel aspects of GR gene expression regulated by the AR signaling in prostate tumors. First, we used patient tumor samples, PCa xenografts and PCa cell lines to demonstrate that AR signaling negatively regulated GR expression. Second, we provided evidence that GR expression did not positively correlated with aggressive tumor phenotypes. Rather, it was inversely associated with activated AR function. Third, we identified a functional ARE within the GR promoter that can be recognized directly by AR to suppress GR gene transcription.

The AR signaling has an inhibitory impact on GR gene transcription. The levels of GR protein were significantly elevated in prostate tumors under ADT, but dramatically dropped when re-activation of AR occurred in CRPC tumors (Fig. 1). These observations were further supported by our xenograft models. All five androgen sensitive SRC tumors possessed strong AR and PSA, but low or no GR expression (Fig. 2). By contrast, strong GR expression was observed in two AR/PSA negative CRPC tumors. Furthermore, castration-resistant C4-2 xenografts expressed higher PSA levels, but lower levels of GR (Fig. 3*b*). Although these castration-resistant C4-2 xenografts sustained scattered colonies of AR positive cells in the presence of AR shRNA, GR expression always appeared in tumor cells that were AR negative (Fig. 3). Additionally, direct suppressive impacts of AR activity on GR gene transcription at the molecular level were consistently observed in a wide panel of PCa cell lines (Figs. 4–6). These results consolidate the conclusion that AR signaling has a general suppressive impact on GR expression.

Our studies do not support that GR expression is positively correlated with aggressive phenotypes of prostate tumors. Under NHT treatment, GR expression was higher in the tumors groups that were still sensitive to ADT, but withdrawal when tumors were at the aggressive CRPC stage (Fig. 1). GR expression is inversely correlated with PSA levels and positively associated with the regressive phenotype of C4-2 xenografts encoding AR shRNA in castrated NUDE mice (Fig. 3). Additionally, tumor doubling time and the capacity of metastasis and local invasion of the SRC tumor models were also not correlated with GR expression (Fig. 2*a*). Furthermore, we also observed PSA negative tumors that were GR positive (Figs. 1*d* and 2*b*), indicating that the function of GR cannot replace AR in driving tumor progression in all prostate cancers, even though both steroid receptors share high homology in their DNA binding domains.

The controversial roles of GR had been reported in several studies and no consensus had been drawn on whether GR functioned as a driver for CRPC progression. Enforced expression of GR in PCa cell lines was shown to regulate a subset of AR targeted genes under androgen-depleted

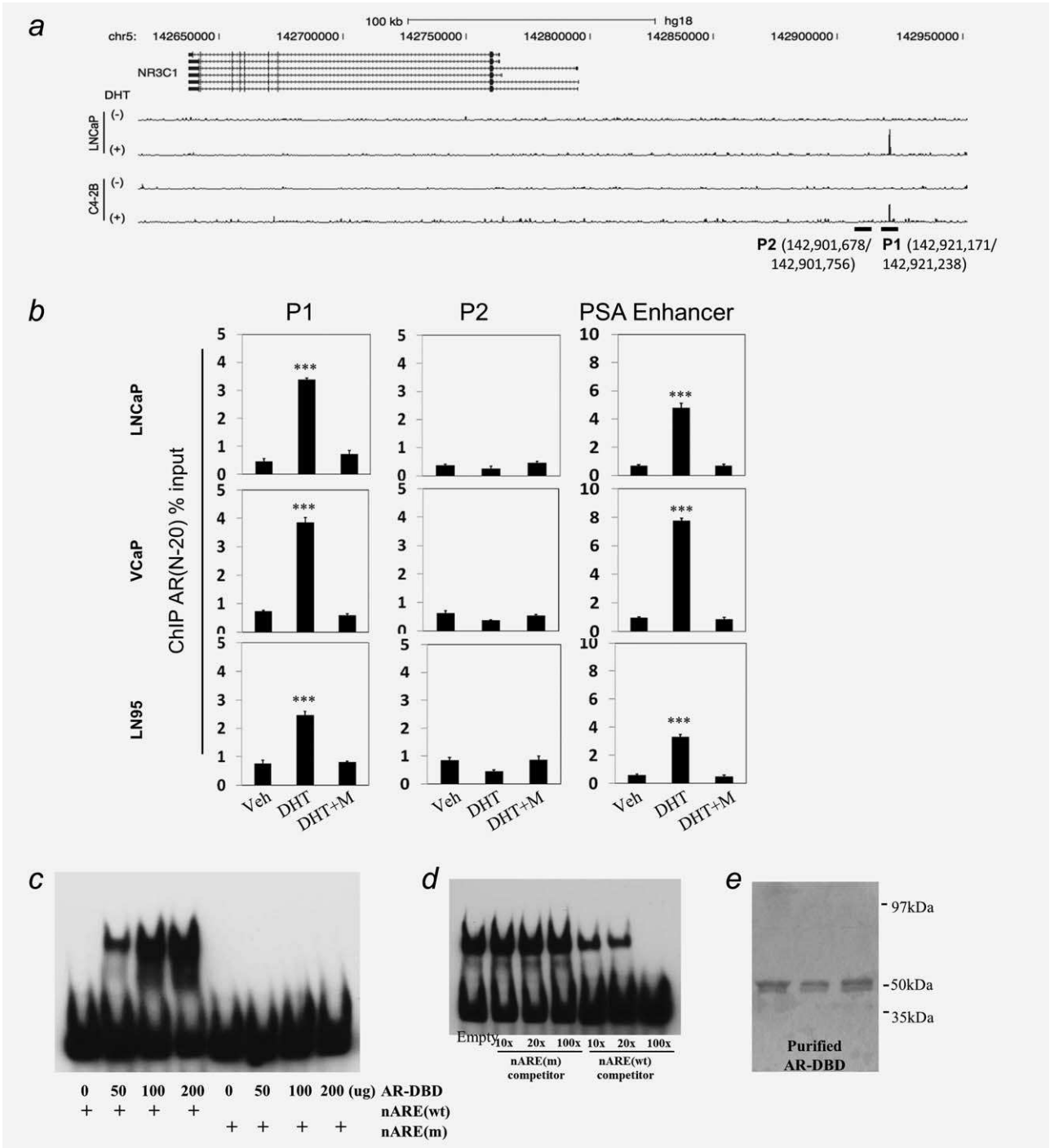

Figure 5. Identification of a nARE in the GR promoter. (*a*) ChIP-seq results showed AR binding to the GR (NR3C1) gene locus after 4 hr DHT treatment in LNCaP and C4-2B cells. Locations of primers used for ChIP assays were marked as P1 and P2. (*b*) LNCaP, VCaP, and LN95 cells were maintained in androgen-depleted medium for 48 hr. ChIP assays were performed using cells treated with vehicle, 10 nM DHT, and/or 25 μM MDV3100 with AR antibody. Precipitated DNA fragment were used as templates to amplify P1 and P2 regions by real-time qPCR and plotted as percentile of input. Data are from five independent experiments and presented as mean ± SEM. Student's *t*-test compared DHT and/or MDV3100 (Enzalutamide) treatment with vehicle treatment with $^{***}p < 0.001$. (*c*) Gel shift assays were performed using purified recombinant AR-DBD protein (0, 50, 100, 200 μg) and biotin labeled DNA probes containing nARE or mutated nARE. (*d*) Gel shift assays were performed by using 100 μg of AR-DBD protein incubated with 2 ng of biotin labeled DNA probe containing nARE. Reactions also contained 0-, 10-, 20-, and 100-fold of non-biotin labeled DNA competitor oligos. (*e*) AR-DBD protein were purified by Glutathione Separose 4B (GE Healthcare) as reported.[29] Protein samples in triplicates were separated on SDS gel and stained by coomassie brilliant blue.

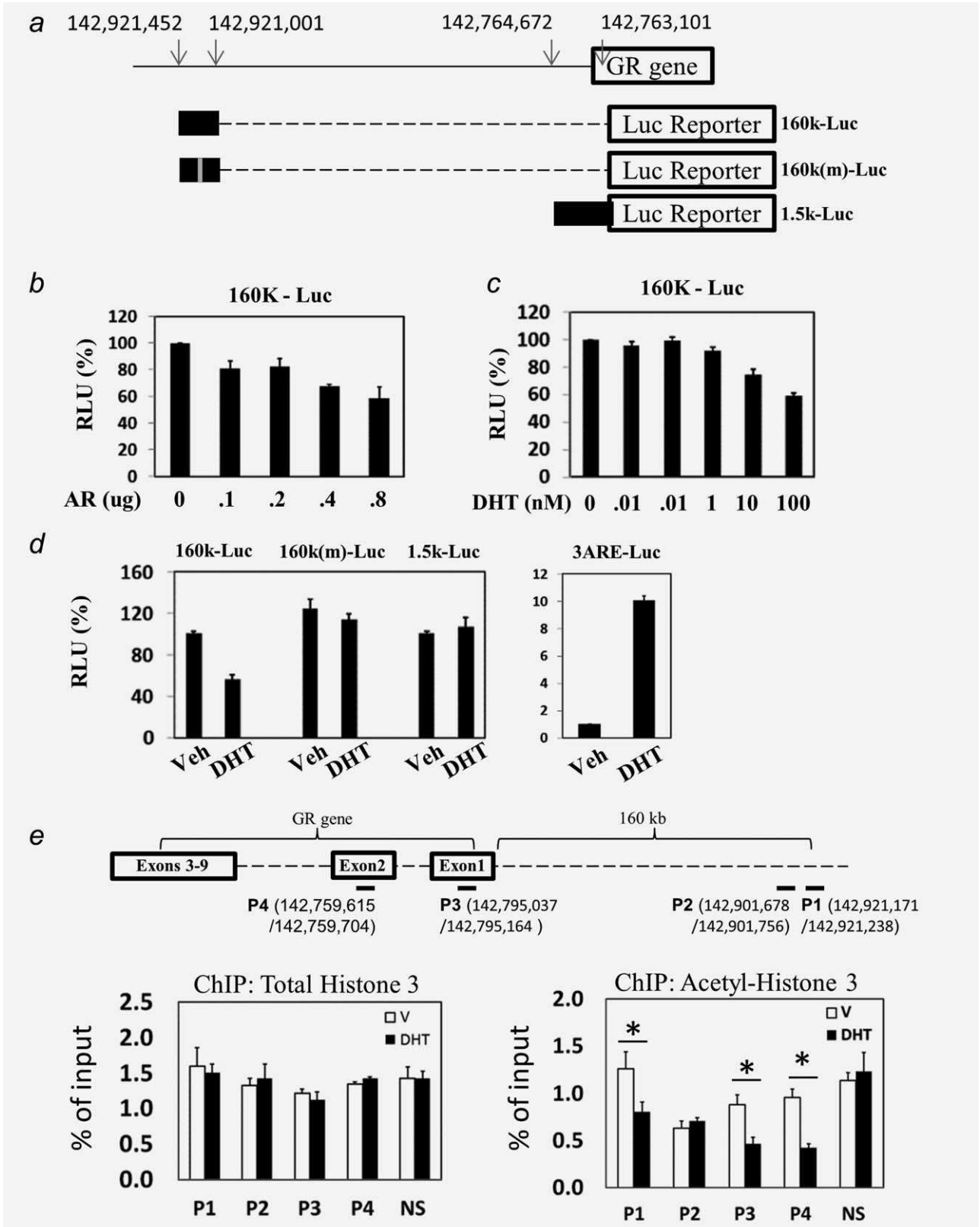

Figure 6. The nARE mediates AR suppression of GR transcription. (*a*) Genomic locations of DNA fragments at or near the GR gene were cloned into a luciferase reporter vector. (*b*) LNCaP cells were maintained in androgen-depleted medium for 48 hr. The 160K-Luc reporter was co-transfected into cells with increasing doses of AR expression plasmid in the presence of 10 nM DHT for 24 hr. (*c*) The 160K-Luc reporter was transfected into LNCaP cells and treated with vehicle or increasing doses of DHT for 24 hr. (*d*) LNCaP cells were transfected with the indicated luciferase reporters and treated with vehicle or 10 nM DHT for 24 hr. Luciferase activities were measured and normalized to Renilla luciferase activities. Values were shown as means + SEM from three independent experiments. (*e*) Schematic diagram of human GR gene with locations of primers used for ChIP assays. LNCaP cells were maintained in androgen-depleted medium for 48 hr. ChIP assays were performed with total Histone 3 or acetylated Histone 3 antibody after either vehicle or 10 nM DHT treatment for 24 hr. ChIP data are derived from three independent experiments with triplicate samples per experiment. Student's *t*-test compared vehicle and DHT treatments with $*p < 0.05$.

conditions.[10,11] Clonal selection of LNCaP xenografts *in vivo* by the anti-androgen Enzalutamide revealed that several tumors gained GR overexpression.[11] Knocking down GR expression by shRNA in these tumors partially diminished Enzalutamide resistance.[11] Additionally, higher GR expression was associated with a shorter tumor take interval in LAPC4 xenografts and quicker tumor progression in CWR-22Rv1 xenografts.[12] However, several studies also reported that glucocorticoid/GR exerted its anti-proliferative function through suppressing TGF-beta and IL-6 signaling *in vivo* and *in vitro*.[31,32] Enhanced GR expression in LNCaP cells suppressed multiple signal pathways (e.g., MAPK) and inhibited cell proliferation and xenograft tumor growth.[33] When cancer stroma was taken into consideration, glucocorticoids also inhibited tumor growth of DU145, but not PC3 and LNCaP xenografts through inhibiting the expression of VEGF and IL-8 to repress angiogenesis.[34] It still remains to be answered on how much bioavailable glucocorticoids in patient tumor tissues can be used to stimulate GR. However, exogenous glucocorticoids suppressed androgen synthesis through a feedback inhibitory mechanism of the hypothalamic/pituitary axis.[35] Additionally, multiple clinical trials have also reported that CRPC patients who received glucocorticoids usually showed serum PSA declines and symptomatic improvement.[14–16] Together, these studies implied that multiple-functional properties of GR signaling exist in PCa. Tumor heterogeneity, paracrine interaction between cancer and its associated stroma, systematic androgen synthesis all should be considered for the functional roles of GR in prostate tumors.

Our results are not consistent with recently published data showing that GR confers Enzalutamide resistance in LNCaP xenografts obtained after clonal selection.[11] This can largely be explained by the heterogeneity of CRPC tumors. Indeed, not all of the Enzalutamide-resistant LNCaP xenografts are GR positive or have GR overexpressed.[11] In our studies, there were CRPC tumors expressing high levels of GR, but no PSA (Figs. 1*d* and 2*b*), indicating that GR does not always function as a substitute for AR in driving AR-targeted gene expression. Many human CRPC tumors gained re-activation of AR signaling post castration therapy through mechanisms including AR gene amplification, gain-of-function mutations or de novo androgen synthesis. However, emerging evidence have also indicated that some other CRPC tumors (e.g., neuroendocrine differentiated tumors) do not express AR or AR regulated genes, but are highly invasive.[22] In addition, tumors expressing lower levels of PSA are often more resistant to androgen deprivation than tumors expressing high PSA levels.[36] These findings indicate that many CRPC tumors are in the process of or have become AR-signaling independent, which could explain why the tumors are poorly responsive to Enzalutamide.[11] Coincidently, GR expression is upregulated due to the suppressive effects of AR being diminished. Irrespective, our results as well as results from previous publications,[11,12] all support the conclusion that AR signaling blockade induces GR expression. However, that GR confers resistance to antiandrogen therapy may not apply to all forms of CRPC tumors.

Our studies demonstrate for the first time the molecular mechanism by which AR signaling directly inhibits GR gene transcription in tumor xenografts, patient tissue samples and PCa cell lines. We have shown that a nARE is the only AR binding site at or near the GR gene (Fig. 5). AR interacts directly with this nARE, reduces histone 3 acetylation of the GR gene and suppresses its downstream GR gene transcription (Figs. 5 and 6). Interestingly, the nARE is 160 kbp upstream of the GR gene. This phenomenon was also observed in studies on the cistrome of other transcription factors including the AR,[37] suggesting that the long-range regulation of gene transcription is a common mechanism. However, our luciferase assay showed that DHT-activated AR only suppressed 40% of expression of a luciferase reporter, a much less repressive effect than endogenous GR gene inhibition by AR. These results suggest that there exist other protein factors or cis-elements located between the nARE and the GR gene that may also contribute to AR inhibition of GR transcription. This may partially explain why AR signaling is not always inversely correlated with GR positivity in some prostate tumors. Such factors were not detected by our luciferase assays. Polycomb group family member, EZH2, had been reported to be recruited by AR to AR targeted genes, subsequently resulting in chromatin condensation and gene transcription silencing.[18] This mechanism was not applicable to GR gene suppression by AR, since knockdown of EZH2 in LNCaP cells did not alter GR protein levels, suggesting other chromatin modifiers or AR corepressors may be involved (data not shown).

In summary, GR expression is negative regulated by the AR signaling through a nARE in GR gene promoter. GR may be used as a marker to monitor the AR signaling.

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