

Maintenance of Androgen Receptor Inactivation by S-Nitrosylation

Yu Qin, Anindya Dey, Hamsa Thayerle Purayil, and Yehia Daaka

Abstract

Antiandrogens target ligand-binding domain of androgen receptor (AR) and are used as first-line therapeutics to treat patients diagnosed with locally advanced and metastatic prostate cancer. Although initially beneficial as judged with actual tumor mass shrinkage, this therapy invariably fails and the cancer reappears as castration-resistant disease. Here, we report that increased intracellular nitric oxide (NO) levels lead to growth inhibition of both androgen-dependent and castration-resistant prostate tumors through a mechanism that involves AR function inactivation by S-nitrosylation of a single C601 residue present in the DNA-binding domain. AR S-nitrosylation does not impact its subcellular distribution but attenuates its ability to bind AR-responsive elements in promoter region of target genes. Mechanistically, AR is transnitrosylated by its partner HSP90 protein. Ubiquitous small-molecule NO donors promote the AR S-nitrosylation and inhibit growth of castration-resistant prostate tumors. These findings reveal a new mechanism of regulating AR function and suggest that sequential targeting of distinct domains of AR may extend therapeutic efficacy for patients with advanced prostate cancer. *Cancer Res*; 73(22); 6690–9. ©2013 AACR.

Introduction

Prostate cancer is the most diagnosed malignancy in males and the second leading cause of male cancer-related mortality in majority of Western countries (1). Patients with cancer confined to the prostate gland have several treatment options, including watchful waiting, surgery, and radiation. Pathologic growth of the prostate is controlled mainly by steroid androgens and locally advanced and metastatic diseases are treated with endocrine therapies aimed to decrease circulating androgen levels via castration, or block androgen receptor (AR) activation with ligand antagonist antiandrogens (2). A limitation of the hormonal therapies is that they offer only a temporary relief in that the disease progresses to castration-resistant prostate cancer (CRPC) characterized by aggressive growth and invasion of distal organs, predominantly the bone (3–5) and to date, there is no cure for CRPC.

Factors involved in the transition of prostate cancer from androgen-dependent to CRPC are not well established and present an obstacle to improving disease outcome. Emerging evidence implicates AR not only in the initiation but also progression of the disease to CRPC. Available work has iden-

tified activating mutations in AR, and model transgenic mice harboring mutated AR develop prostatic intraepithelial neoplasia that progress to invasive cancer (6). CRPC lesions exhibit frequent mutations in AR, which allow it to be activated by steroids other than androgens, or even antiandrogens. Also, AR can be transactivated by factors other than androgens, including peptide growth factors (7) that exert their effects on target cells by activating cognate plasma membrane-anchored receptors. These laboratory and clinical results firmly implicate AR in prostate carcinogenesis.

AR is an androgen-dependent transcription factor (8) that manifests its effects on target cells by controlling expression of specific genes through association with protein coregulators in the form of coactivators and corepressors (9, 10). In general, multi-protein complex formation is controlled by posttranslational changes of partner proteins, and AR has been reported to undergo several posttranslational modifications, including phosphorylation, ubiquitylation, and acetylation that impact its subcellular localization, stability, and overall transcriptional activity (11–13). Here, we tested the idea that AR undergoes the S-nitrosylation modification, that is, covalent attachment of a nitric oxide (NO) group to a cysteine thiol, and determined the effect on AR signal transduction and consequent growth of prostate tumors.

Materials and Methods

Cell culture and manipulations

LNCaP, PC3, 22Rv1, and HEK293 cells were obtained from American Type Culture Collection. LNCaP C4-2 cells were obtained from MD Anderson Cancer Center (Houston, TX). LNCaP and 22Rv1 cells were maintained in RPMI-1640 supplemented with 10% FBS, 1% HEPES, 1% D-glucose, and 1% sodium pyruvate. LNCaP C4-2 cells were cultured in DMEM/Ham's F12

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supplemented with 5% FBS, 5 $\mu\text{g}/\text{mL}$ insulin, 13.65 pg/mL triiodothyronine, 5 $\mu\text{g}/\text{mL}$ apo-transferrin, 0.244 $\mu\text{g}/\text{mL}$ α -biotin, and 25 $\mu\text{g}/\text{mL}$ adenine (all from Sigma). PC3 and HEK293 cells were maintained in Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% FBS. Androgen deprivation was accomplished by incubation in phenol red-free culture medium supplemented with 5% charcoal-stripped serum. Stable overexpression of endothelial nitric oxide synthase (eNOS) in LNCaP cells was achieved by transfecting cells with pcDNA3-HA-eNOS. Transfected cells were propagated in the presence of G418 (1 mg/mL) for selection for 4 weeks. Positive clones were pooled and maintained with G418 (100 $\mu\text{g}/\text{mL}$). Transient forced overexpression of AR in PC3 cells was done by transfecting cells with pcDNA3-Flag-AR. Stable knockdown of eNOS was achieved by infection of short hairpin RNA (shRNA) constructs (Open Biosystems) in lentiviral pLKO vector plus equal concentration of vesicular stomatitis virus G and $\Delta 8.9$ vector into packaging HEK293T cells. Lentivirus-containing medium was harvested, mixed with polybrene, and used to infect LNCaP cells. The infected polyclonal cells were selected with 2 $\mu\text{g}/\text{mL}$ puromycin for 2 weeks and maintained in medium containing 1 $\mu\text{g}/\text{mL}$ puromycin. Cell growth and doubling time analyses were done with WST-1 reagent.

Real-time PCR

Cells were cultured in starvation medium for 24 hours followed by stimulation with R1881 (1 nmol/L) in the presence, or absence, of S-nitrosoglutathione (GSNO) for 48 hours. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) and mRNA was reverse-transcribed to cDNA using the iScript Reverse Transcription Supermix (Bio-Rad). Prostate-specific antigen (PSA; in LNCaP) and FKBP51 (in PC3) gene levels were measured by iQ SYBR Green Supermix (Bio-Rad) and iQ5 thermal iCycler detection system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences were as follows: PSA (sense) 5'-AGA ATC ACC CGA GCA GGT GC-3'; PSA (antisense) 5'-CAG AGG AGT TCT TGA CCC CA-3'; FKBP51 (sense): 5'-CCA AAG CTG TTG AAT GCT GTG A-3'; FKBP51 (antisense): 5'-CAA ACT CGT TCA TGA GC A GCT G-3'; GAPDH (sense) 5'-CAT GGG TGT GAA CCA TGA GAA-3'; GAPDH (antisense) 5'-GGT CAT GAG TCC TTC CACGAT-3'.

Western blotting

Appropriately treated cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and fractionated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and immunoblotted with the indicated primary antibody (used at a 1:1,000 dilution except anti- β -actin antibody that was used at a 1:5,000 dilution) at 4°C overnight. Horseradish peroxidase-conjugated anti-mouse (1:30,000 dilution) or anti-rabbit (1:20,000 dilution) secondary antibodies were used and proteins were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Biotin switch assay

Protein S-nitrosylation reactions were performed exactly as described before (14, 15). For protein transnitrosylation,

purified recombinant HSP90 β and AR were dialyzed into Hepes-EDTA-neocuproine buffer. Equimolar amounts of each protein were exposed, or not, to CysNO (100 $\mu\text{mol}/\text{L}$) for 30 minutes at 37°C in the dark. The resultant S-nitrosylated proteins were desalted and then used as NO donors to their un-nitrosylated purified protein counterpart. Resultant samples were subjected to the NO-biotin switch assay to monitor transnitrosylation of each protein.

Chromatin immunoprecipitation

Cells were subjected to chromatin immunoprecipitation (ChIP) analysis using the Chromatin Immunoprecipitation Kit (Millipore), according to the manufacturer's instructions. Anti-AR (clone N20) or equal amount of isotype control antibody was added to purified DNA and incubated overnight at 4°C with rotation. Protein G agarose beads were added to pellet protein-DNA complexes followed by sequential washing once with ice-cold low salt, once with high salt, once with LiCl, and twice with Tris-EDTA buffers. DNA-protein cross-link was reversed and chromatin DNA was purified and measured by PCR. Primers were as followed: PSA promoter (sense) 5'-TCT GCC TTT GTC CCC TAG AT-3'; PSA promoter (antisense) 5'-AAC CTT CAT TCC CCA GG ACT-3'; FKBP51 promoter (sense) 5'-GAG CCT CTT TCT CAG TTT TG-3'; FKBP51 promoter (antisense) 5'-CAA TCG GAG TGT AAC CAC ATC-3'. DNA products were used for quantitative PCR analyses by iQ SYBR Green Supermix (Bio-Rad) and iQ5 thermal iCycler detection system or separated on agarose gel, stained with ethidium bromide, and visualized under UV light.

Electromobility shift assay

Sequence of the FKBP51 consensus binding site oligonucleotides was 5'-ACA TTC AGA ACA GGG TGT TCT GTG CTC-3'. Double-stranded oligonucleotides (100 ng) were end-labeled with [γ -³²P]-ATP (specific activity, 3,000 Ci/mmol) using polynucleotide kinase. For the electromobility shift assay (EMSA) reaction, 1 ng of labeled probe was mixed with 5 μg of nuclear extracts in 20 μL EMSA buffer containing 20 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 5 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 μg poly(dI-dC), and 8% (v/v) glycerol. Anti-AR-N20 (0.5 μg) antibody (Santa Cruz Biotechnology) was used for detecting AR-ARE complex supershift.

Animal studies

Severe combined immunodeficient (SCID) mice were purchased from Harlan Laboratories. The Institutional Animal Care and Use Committee preapproved all animal procedures. LNCaP cells (10^6 in 50 μL phenol red-free RPMI-1640) were mixed with equal volume of Matrigel and the mixture was inoculated subcutaneously into the right flank region of mice. Tumor dimensions were determined with caliper and tumor volume (v) was estimated using the equation $v = \pi/6 \times L \times S^2$, where L is the long and S the short lengths of the tumor mass. Tumor growth rate was measured weekly. When tumor size reached 100 to 200 mm^3 , animals were randomly divided into three treatment groups: (i), Control; (ii), luteinizing hormone-releasing hormone (LHRH); and (iii), GSNO. Control mice were injected with vehicle PBS. Animals were treated daily with

LHRH (100 µg/kg body weight) subcutaneously for 14 days, or with GSNO (10 mg/kg body weight) intraperitoneally for 7 days. At the endpoint, mice were sacrificed and tumor tissues were dissected and analyzed for PSA levels by Western blotting. Experiments were repeated three times and each datapoint represents values from 5 to 10 animals.

Results

Elevated NO expression attenuates prostate tumor growth

Deregulated NO levels have been implicated in human disease, most notably those of the cardiovascular and immune systems. Less, however, is appreciated about potential role of ubiquitous NO in tumorigenesis (16). NO has been reported to exert pro- and antigrowth effects in a manner that is dependent upon cellular localization and expression level of NOSs and exposure duration to NO (17). NOSs expression is increased in prostate cancer (18), and increased expression of eNOS in prostate cancer tissues highly correlates with decreased patient survival (19). Impact of elevated expression of NOSs or NO in CRPC remains unclear. We determined effect of model NO-donor diethylenetriamine NONOate (DETA-NO) on the growth rate of prostate cancer cells. Growth of androgen-dependent LNCaP (Fig. 1A) as well as castration-resistant 22Rv1 (Fig. 1B) cells that endogenously express AR was significantly attenuated in response to treatment with DETA-NO. Similar treatment of PC3 cells that do not express AR with DETA-NO for up to 24 hours did not evidence a statistically significant effect on the cell proliferation (Fig. 1C), consistent with previous results (20). Treatment of PC3 cells with DETA-NO for 48 hours, however, exhibited a significant decrease in the cell proliferation. The treatment with DETA-NO showed no effect on the LNCaP cell apoptosis, as assessed with PARP cleavage (Supplementary Fig. S1). These results support the conclusion that, at concentrations used here, DETA-NO targets the proliferation machinery of prostate cancer cells.

To test that intracellular NO impacts the cell growth, we generated a LNCaP cell line that stably overexpressed eNOS (LNCaP-eNOS; Supplementary Fig. S2A). LNCaP-eNOS cells evidenced a prolonged doubling time of 95.7 hours, in comparison with the faster doubling time of 55.2 hours for the control empty-vector (LNCaP-EV) cells (Fig. 1D). Conversely, the stable knockdown of endogenous eNOS expression with shRNA (LNCaP-sheNOS; Supplementary Fig. S2A) accelerated the cell doubling time to 22.7 hours (Fig. 1D). Moreover, androgen-induced proliferation of LNCaP-eNOS cells was significantly reduced compared with LNCaP-EV cells (Fig. 1E). Together, these results establish exogenously supplied and endogenously produced NO as proliferation suppressor of AR-expressing prostate cancer cells.

Effects of modulating NO levels on LNCaP tumor growth in animals were measured next. LNCaP-EV, LNCaP-eNOS, and LNCaP-sheNOS cells were inoculated subcutaneously in the flanks of SCID mice. Results show that while knockdown of endogenous eNOS accelerated the tumor growth rate, forced overexpression of eNOS inhibited it, in comparison with control LNCaP-EV tumors (Fig. 2A). Analysis of tumor tissue

showed that expression of AR-controlled PSA was reduced in LNCaP-eNOS, but increased in LNCaP-sheNOS samples (Supplementary Fig. S2B), implying an effect of endogenously synthesized NO on AR function.

GSNO inhibits castration-resistant prostate tumor growth

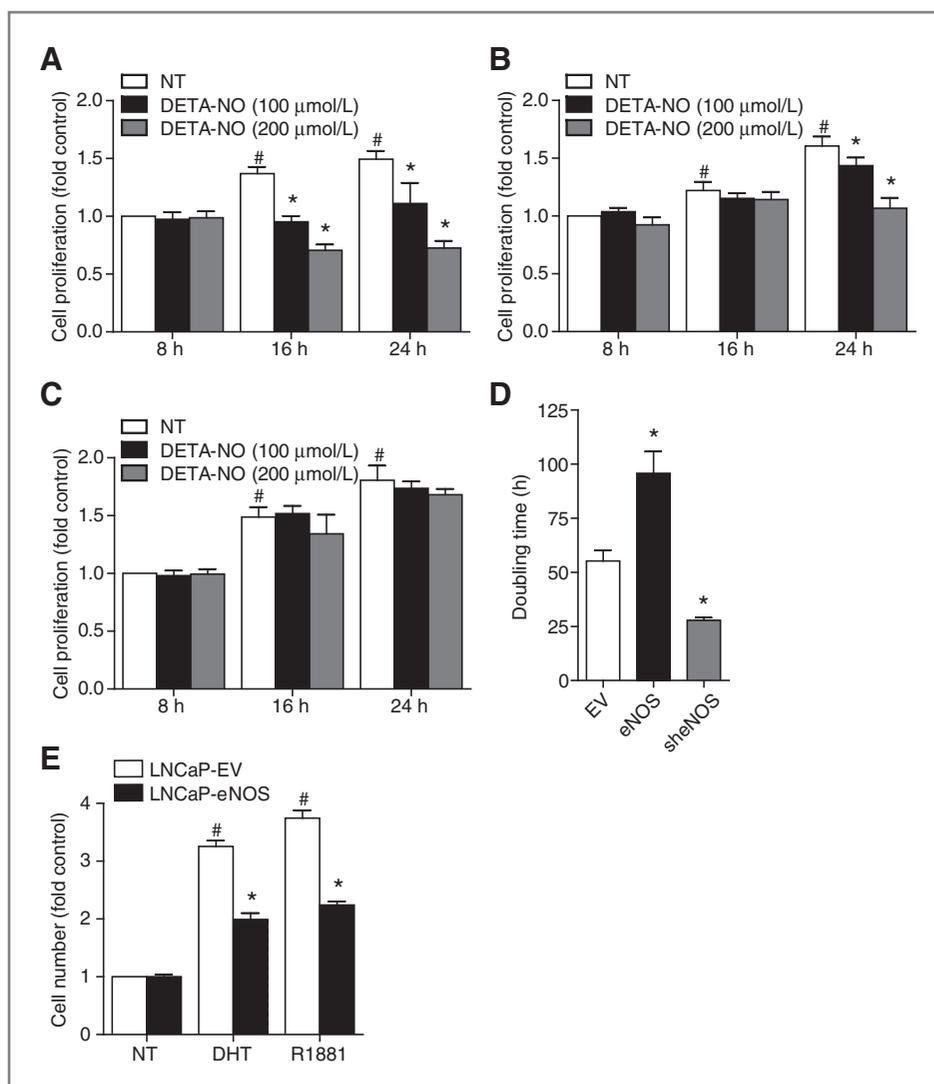
We used LNCaP and LNCaP-derived C4-2 cells as models of androgen-dependent and ensuing castration-resistant, respectively, prostate cancer to elucidate possible distinguishing effects of NO on the tumor growth. Tumor-bearing SCID mice were randomly divided into three groups that were treated with vehicle (Veh), LHRH, the first-line hormone deprivation therapy for advanced prostate cancer, or GSNO, the most abundant form of physiologic NO-donor in mammalian cells (21). Consistent with clinical outcomes, treatment with LHRH suppressed growth of parental LNCaP tumors but showed no effect on the growth rate of LNCaP C4-2 tumors (Fig. 2B). Remarkably, the treatment with GSNO inhibited growth of both LNCaP and LNCaP C4-2 tumors (Fig. 2B), but showed no effects on the overall health of animals as measured with intact body weight and physical activity. Furthermore, pathology review of heart, lung, spleen, and liver tissue sections indicated lack of GSNO toxicity, consistent with previous results (22). Cotreatment of animals harboring LNCaP C4-2 tumors with LHRH and GSNO did not further decrease the tumor growth rate in comparison with treatment with individual LHRH or GSNO, suggesting they act upon common effector(s). These results establish that endogenously produced as well as exogenously added NO inhibits growth of both androgen-dependent and castration-resistant prostate tumors.

NO attenuates AR activity

LNCaP cell growth is dependent upon AR signaling, and we tested the idea that NO impacts AR activity. Androgen-induced activation of endogenous AR, as measured with PSA expression, was significantly impaired upon treatment with escalating doses of GSNO (Fig. 3A). Confirmatory results showed that androgen-induced AR activation, as measured with AR-response element (ARE)-controlled luciferase reporter, was also reduced in LNCaP cells treated with GSNO or DETA-NO (Supplementary Fig. S3A), and mirroring results were obtained in LNCaP-eNOS cells (Supplementary Fig. S3B). Concordantly, the knockdown of endogenous eNOS expression increased PSA levels, but had no effect on total AR protein expression (Fig. 3B). These results suggest that NO represses the inducible activation of AR in prostate cancer cells.

AR-null prostate cancer PC3 cells were used to illustrate whether AR is directly regulated by NO. AR activity was measured by detection of endogenous AR-regulated *FKBP51* gene. *FKBP51* mRNA was detected only in cells expressing AR and treated with R1881 (Fig. 3C), justifying use of this assay to measure AR activation. Significantly, the androgen-induced *FKBP51* mRNA levels were dose-dependently decreased upon treatment with GSNO in PC3-AR cells (Fig. 3C), suggesting the effect of NO is directly through modulating AR signaling. Similarly, PSA gene levels were attenuated in LNCaP cells treated with GSNO (Fig. 3D).

Figure 1. Effect of NO and eNOS on prostate cancer cell growth. Effect of DETA-NO on LNCaP (A), 22Rv1 (B), and PC3 (C) cell proliferation. Equal number of cells was incubated for 24 hours in appropriate culture medium containing 10% FBS in the presence or absence of DETA-NO. For each time point, viable cells were determined after mixing with WST-1 and data are expressed as fold change in absorbance at A_{450} , for which the value of untreated cells that were incubated for 8 hours was defined as 1. *, $P < 0.05$ compared with corresponding nontreated (NT) control cells, and #, $P < 0.05$ compared with nontreated control cells at 8 hours. D, doubling times of LNCaP cells stably overexpressing eNOS or sheNOS were calculated by counting viable cells at 24 hours interval. *, $P < 0.05$ compared with control LNCaP-EV cells. E, forced overexpression of eNOS attenuates the androgen-induced LNCaP cell proliferation. LNCaP-eNOS and LNCaP-EV cells were treated with DHT (10 nmol/L) or R1881 (1 nmol/L) for 96 hours and viable cell numbers were counted. *, $P < 0.05$ compared with control LNCaP-EV cells; #, $P < 0.05$ compared with corresponding LNCaP-eNOS cells. In all panels, the Tukey *post hoc* test following a one-way ANOVA was performed to compare groups. All results represent three independent experiments.



Activated AR translocates to the nucleus and binds to ARE in the regulatory regions of AR-target genes. Treatment with GSNO (Supplementary Fig. S4A and S4B) or forced overexpression of eNOS (Supplementary Fig. S4C) showed little effect on the androgen-induced AR nuclear translocation in LNCaP cells, consistent with previous results (20). However, the forced overexpression of eNOS (Fig. 3E), like the treatment with GSNO (Fig. 3F and Supplementary S5A and S5B), significantly reduced the androgen-mediated AR binding to ARE in the PSA promoter, as determined by ChIP. Also, GSNO dose-dependently inhibited the basal AR binding to PSA ARE in LNCaP cells (Fig. 3G), confirming that NO inhibits AR activity through interference of its binding to promoter elements in target genes.

AR modification by S-nitrosylation

We hypothesized that NO regulates AR activity through S-nitrosylation that involves the covalent attachment of NO group to a free cysteine thiol, and protein S-nitrosylation has

been suggested to mediate NO effects on target cells (21, 23, 24). Biotin switch assay (25) was performed using prostate cancer cells treated with escalating doses of NO-donor S-nitrosocysteine (CysNO), a cell-permeable and byproduct of endogenous GSNO (26). Results establish the posttranslational S-nitrosylation of AR in both LNCaP (Fig. 4A) and 22Rv1 (Fig. 4B) cells. In addition to full-length AR, the 22Rv1 cells express splice-variants that encode truncated proteins lacking ligand-binding domain (AR-V) and our results show that both forms of AR were modified by S-nitrosylation (Fig. 4B). Remarkably, the S-nitrosylation of AR inversely correlates with its transcriptional activity and is regulated by androgen: treatment with R1881 promoted a reciprocal decrease (2-fold) of AR S-nitrosylation content and increase (3-fold) of PSA levels (Supplementary Fig. S6A). Forced overexpression of eNOS resulted in more basal level SNO-protein expression (Supplementary Fig. S6B) in comparison with control LNCaP-EV cells, which was further supported by the measurement of SNO-AR in LNCaP tumors (Supplementary Fig. S6C).

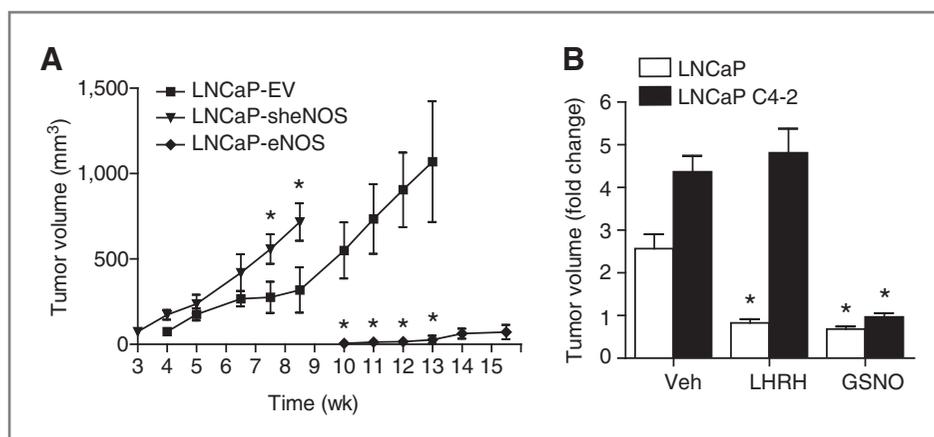


Figure 2. Impact of eNOS expression and treatment with GSNO on prostate tumor growth. **A**, effect of modulating eNOS levels on LNCaP tumor growth. LNCaP-EV, LNCaP-eNOS, and LNCaP-sheNOS cells were inoculated subcutaneously into SCID mice and tumor volumes were measured weekly using a caliper. Each point represents the average tumor size from 5 to 10 animals. *, $P < 0.05$ compared with corresponding control (EV) tumor volume. **B**, GSNO inhibits LNCaP C4-2 tumor growth. LNCaP and LNCaP C4-2 cells were inoculated subcutaneously into SCID mice. When tumors formed (about 100 to 200 mm³), randomly grouped animals were treated daily with LHRH (100 μg/kg body weight) subcutaneously for 14 days, GSNO (10 mg/kg body weight) intraperitoneally for 7 days, or vehicle PBS. Tumor volume changes were measured and compared with values before treatment. A Bonferroni *post hoc* test following a two-way ANOVA was performed to compare the groups. *, $P < 0.05$ compared with corresponding control (Veh) tumors. All results represent three independent experiments.

To identify the cysteine residue(s) corresponding to S-nitrosylation site(s) of AR, S-nitrosylation site identification (27) was performed. Purification efficiency of AR protein was confirmed by Coomassie blue staining (Supplementary Fig. S7A) and Western blotting (Supplementary Fig. S7B), and purified AR protein S-nitrosylation was demonstrated using the biotin switch assay (Supplementary Fig. S7C). Liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis (22, 28) revealed three cysteine residues (C518, C601, and C784) as putative S-nitrosylation sites in AR (Supplementary Fig. S7D). Replacement of the individual residues with serine followed by subjugation of the mutated AR protein to biotin switch assay indicated C601 as the primary candidate S-nitrosylation site in AR (Fig. 4C). Notably, both full-length AR and AR-V proteins contain the C601 residue and are modified by S-nitrosylation (Fig. 4B).

We hypothesized that the mutation of S-nitrosylated cysteine residue in AR increases AR activity. However, androgen-induced *FKBP51* gene expression confirmed that C601S mutation abolished AR activation (Fig. 4D). C601 resides in the second zinc finger of AR DNA-binding domain (DBD), and mutated C601S AR lacked transcriptional activity (Fig. 4D), most likely as a result of its inability to coordinate Zn⁺² and bind promoter elements in target genes. In addition to cysteine thiol, Zn⁺² may coordinate two cysteine thiols and two histidine imidazole nitrogens to form a transcriptionally competent zinc finger domain (29) and we replaced C595 and C601 with histidines (C595/601H) in an attempt to maintain AR-binding ability to ARE. ChIP assays were performed using AR-null PC3 cells ectopically expressing wild-type or C595/601H mutated forms of AR (Fig. 5A and Supplementary Fig. S8A). Treatment with GSNO significantly reduced the wild-type AR binding to ARE (in *FKBP51* promoter), and the binding of mutated C595/601H AR form was absent. We used EMSA to confirm the effect of GSNO treatment on AR binding to ARE

using nuclear extracts from the PC3 cells expressing wild-type or C595/601H forms of AR. Mirroring the findings of the ChIP assay (Fig. 5A), results show that treatment with GSNO significantly decreased the binding of AR to ARE and, again, the binding of C595/601H AR was absent (Fig. 5B and Supplementary Fig. S8B). In agreement with these results, treatment with GSNO impaired ability of estrogen receptor (ER) to bind EREs and consequent gene transcription (30) and the S-nitrosylation of p50 subunit of NF-κB inhibited its binding to target DNA (31). Together, these findings evidence the AR S-nitrosylation that, in turn, impairs its ability to bind ARE, which is required for target gene expression.

HSP90 transnitrosylates AR

A remaining question is just how NO promotes the AR S-nitrosylation. One possibility is that NOS directly binds to AR, leading to increased local NO levels and AR S-nitrosylation. Coimmunoprecipitation experiments of endogenous AR and eNOS (that is expressed in LNCaP cells at higher levels than inducible NOS or neuronal NOS) proteins disputed this idea. Another possible mechanism is that eNOS regulates AR S-nitrosylation indirectly through the binding of common partner HSP90 protein that has been shown to activate eNOS (32) and to be S-nitrosylated in endothelial cells (33). In epithelial prostate cells, HSP90 associates with AR and this complex is maintained in the cytoplasm. Coimmunoprecipitation results confirmed that HSP90β (the constitutively expressed form of HSP90) forms a complex with both AR and eNOS (Fig. 6A). Recent reports have suggested a novel mechanism of NO group transfer between partner proteins as a general signal transduction mechanism, termed transnitrosylation (34–36). We hypothesized that AR becomes S-nitrosylated as a result of NO transfer from S-nitrosylated HSP90. To test this idea, we incubated purified AR or HSP90β proteins with CysNO to form SNO-AR or SNO-HSP90β, respectively. To measure potential

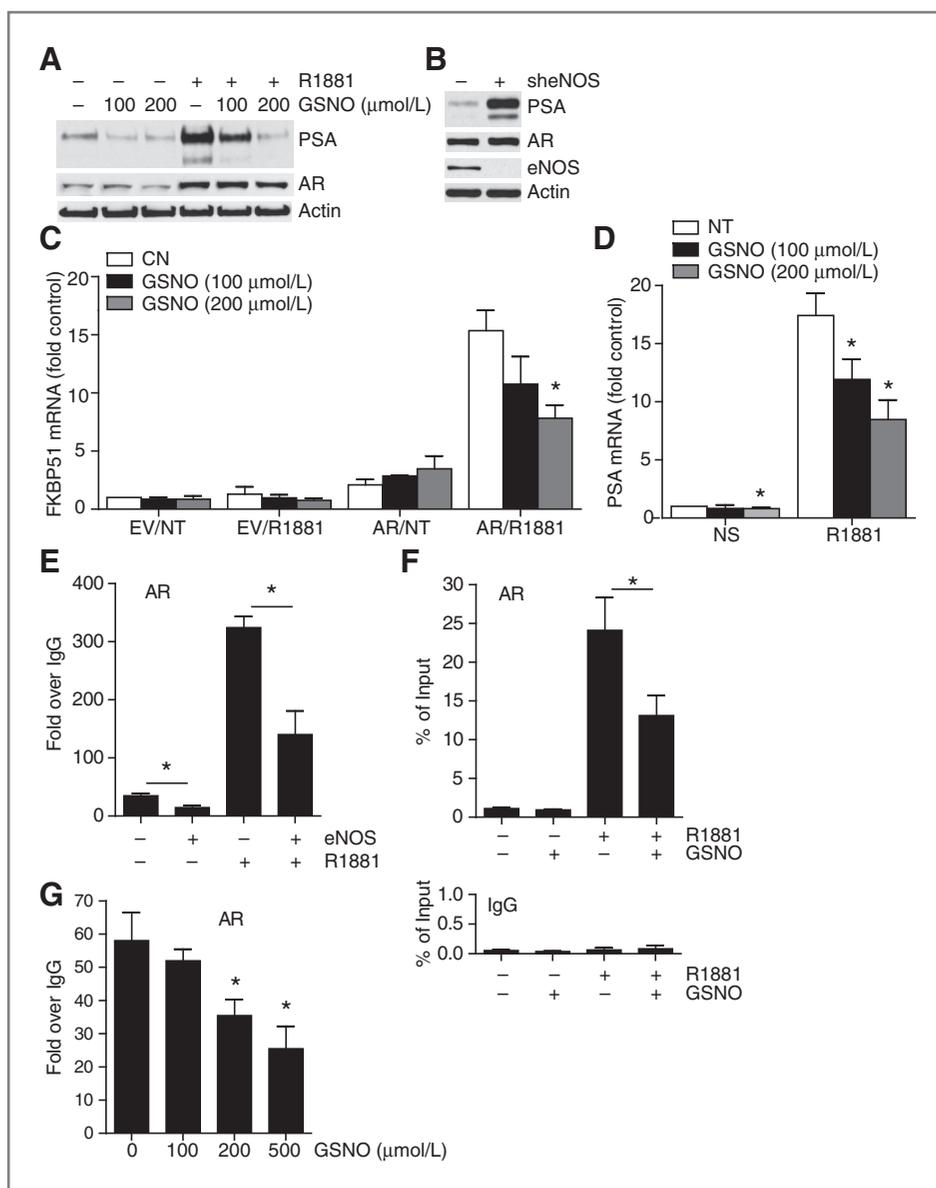


Figure 3. NO regulates AR activity. **A**, GSNO attenuates PSA expression. LNCaP cells were stimulated, or not, with R1881 (1 nmol/L) in the presence or absence of GSNO for 48 hours. Cell lysates were fractionated on SDS-PAGE and proteins were immunoblotted with the indicated antibodies. **B**, knockdown of eNOS expression increases PSA levels. Lysates from GFP (-) or sheNOS (+) LNCaP cells were fractionated on SDS-PAGE and proteins were immunoblotted with the indicated antibodies. **C**, GSNO inhibits R1881-induced FKBP51 gene expression. PC3 cells were transfected with pcDNA3 empty-vector (EV) or pcDNA3-Flag-AR (AR) cDNAs and treated, or not (NT), with R1881 (100 pmol/L) in the presence or absence (CN) of GSNO for 48 hours. FKBP51 mRNA levels were determined by real-time PCR. A Tukey *post hoc* test following a one-way ANOVA was performed to compare groups. *, $P < 0.05$ compared with R1881-stimulated PC3-AR cells without GSNO treatment. **D**, GSNO inhibits PSA mRNA expression. LNCaP cells were treated with GSNO for 48 hours in the presence or absence of R1881 (1 nmol/L). PSA mRNA levels were determined by quantitative PCR, as described in **C**. *, $P < 0.05$ compared with corresponding nontreated cells. **E**, forced overexpression of eNOS attenuates AR binding to ARE (of PSA promoter). Control LNCaP-EV (-) and LNCaP-eNOS (+) cells were starved for 24 hours and then stimulated with R1881 (1 nmol/L) overnight. ChIP assays using anti-AR or control immunoglobulin G (IgG) antibodies were performed. Amplified DNA was subjected to quantitative analysis by PCR. **F**, GSNO suppresses androgen-induced AR binding to PSA ARE. LNCaP cells were treated with GSNO (100 μmol/L) for 24 hours, followed by overnight stimulation with R1881 (1 nmol/L). Cell lysates were subjected to ChIP analysis using anti-AR (top) or isotype IgG (bottom) antibodies. Precipitated DNA was subjected to quantitative PCR amplification. Results are presented as percentages of the input samples. **G**, GSNO suppresses AR binding to PSA ARE. LNCaP cells were treated with increasing doses of GSNO (0–500 μmol/L) for 48 hours, followed by ChIP, as described in **E**. For **E–G**, *, $P < 0.05$.

transnitrosylation, each of these SNO-proteins was mixed with their unlabeled counterpart, that is, SNO-HSP90β with AR (Fig. 6B), or SNO-AR with HSP90β (Fig. 6C). We performed

the biotin switch assay and found that AR was transnitrosylated by SNO-HSP90β (Fig. 6B). Distinctly, SNO-AR was unable to transnitrosylate HSP90β (Fig. 6C), implying that the transfer

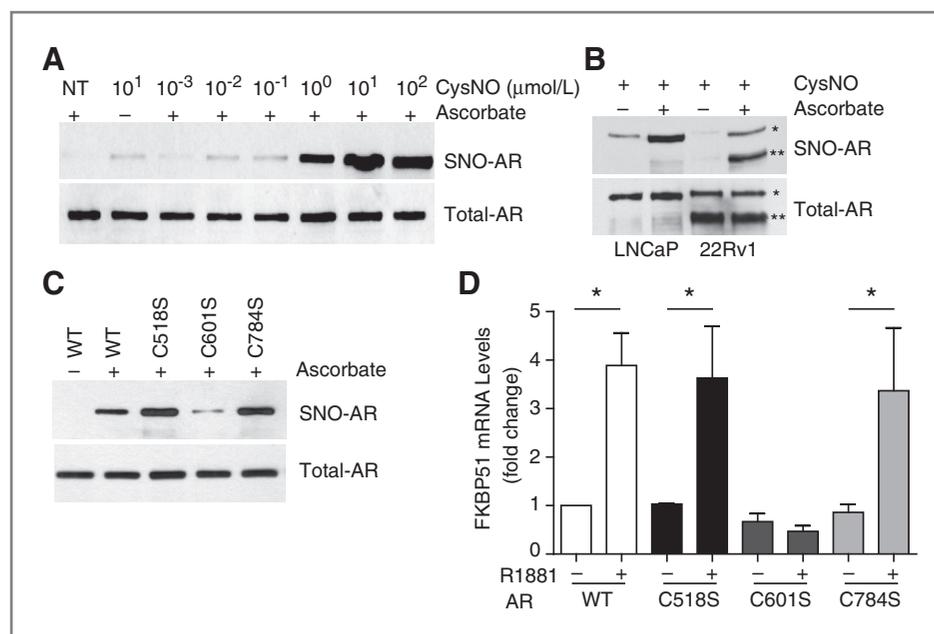


Figure 4. AR is S-nitrosylated on C601. **A**, S-nitrosylation of AR. LNCaP cells were lysed and mixed with escalating doses of CysNO and AR S-nitrosylation was determined with biotin switch assay (top). Equal amounts of cell lysate were immunoblotted with anti-AR antibody to show the equal protein loading (bottom). **B**, S-nitrosylation of endogenous AR. LNCaP and 22Rv1 cells were lysed and mixed with CysNO (100 $\mu\text{mol/L}$) and AR S-nitrosylation was determined with biotin switch assay (top). Equal amounts of cell lysate were immunoblotted with anti-AR antibody to show the equal protein loading (bottom). *, full-length AR; **, AR splice variant that lacks ligand-binding domain. **C**, S-nitrosylation of AR is localized to the C601 residue. AR-null HEK293 cells were transiently transfected with cDNAs encoding Flag-AR [wild-type (WT), C518S, C601S, or C784S] and subjected to S-nitrosylation analysis as described in **A**. **D**, AR C601S is devoid of transcriptional activity. PC3 cells were transfected with pcDNA3-Flag-AR (WT, C518S, C601S, and C784S), starved for 24 hours and then stimulated, or not, with R1881 (1 nmol/L) for 16 hours. FKBP51 mRNA levels were measured by real-time PCR and normalized by GAPDH and AR levels. A Tukey *post hoc* test following a one-way ANOVA was performed to compare groups. *, $P < 0.05$ in comparison with corresponding controls not treated with R1881.

of NO is unidirectional. The transfer of NO from SNO-HSP90 β to AR was observed using purified proteins, suggesting the reaction is thermodynamically favorable.

We examined feasibility of AR transnitrosylation by SNO-HSP90 β under *in situ* conditions. PC3 cells ectopically expressing Flag-AR alone (Fig. 6D) or together with Myc-HSP90 β (Fig. 6E) were treated, or not, with CysNO, followed by biotin switch assay. Relative levels of SNO-AR and SNO-HSP90 β were used to calculate the relative redox potential ($\Delta E^{\circ'}$) and the associated change in Gibbs free energy ($\Delta G^{\circ'}$), as described previously (35). The results show a $\Delta E^{\circ'}$ of 22.38 ± 3.11 mV and a $\Delta G^{\circ'}$ of -2.16 ± 0.3 kJ/mol, confirming a net energy gain for the transfer of NO from SNO-HSP90 β to AR and supporting the conclusion that the transnitrosylation reaction occurs spontaneously in cells.

Discussion

AR plays a central role in the initiation and progression of prostate cancer and serves as a primary target for developing drugs to treat patients with advanced disease. AR consists of three demarcated domains: a variable N-terminus domain that is involved in receptor transactivation and cofactor recruitment, a centrally located DBD that contains two zinc finger motifs, and a C-terminus that encompasses the ligand binding domain (LBD). Although initially effective in the treatment of

locally advanced and metastatic diseases, antiandrogens that target LBD have failed to achieve sustained inhibition of AR signaling, or to cure prostate cancer (4). Recently developed next generation antiandrogen MDV3100 (37) evidenced added survival benefit to patients with CRPC, but the clinical improvements remain short-lived (4, 38), providing rationale to find other targets in AR to better treat patients with advanced disease.

Metastatic and CRPC lesions express AR deletion mutant forms (collectively referred to as AR-V) that lack the LBD (39, 40), and recent work shows AR-V to be transcriptionally active (41, 42). Emerging clinical outcomes suggest that expression of AR-V is increased in CRPC and metastatic lesions, and may facilitate progression of prostate cancer in patients undergoing hormonal therapy (40, 43). In animals, interference of AR-V activation attenuates growth of model CRPC tumors (41). Hence, in addition to full-length AR, AR-V may serve as drug targets to inactivate the receptor. Our results show that S-nitrosylation of C601, located in AR DBD and shared by full-length AR and AR-V, inhibits AR function and growth of CRPC tumors. The sequential targeting of distinct domains in AR may provide a more sustained benefit to patients diagnosed with metastatic and CRPC diseases.

Our data show that C601 is a primary target of NO, and that S-nitrosylation at this site inhibits AR activity. C601 is located in the second zinc finger motif of the AR DBD and is required

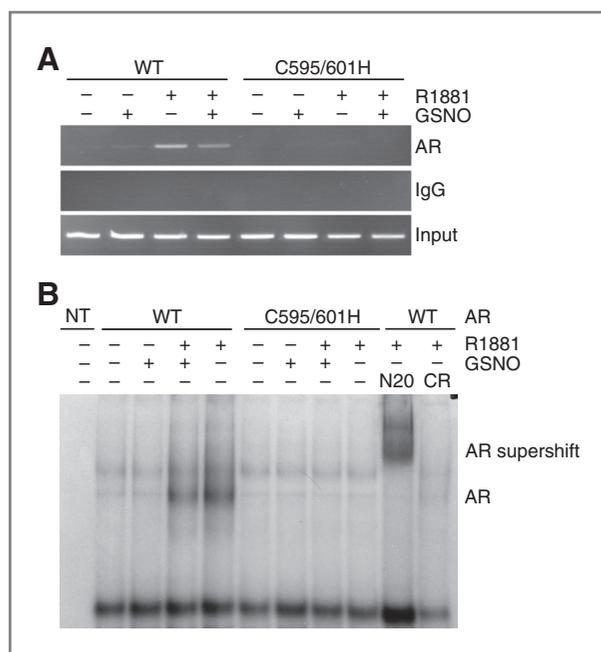


Figure 5. NO attenuates binding of AR to ARE. **A**, AR C595/601H is impaired in binding to ARE. PC3 cells ectopically expressing Flag-AR [wild-type (WT), C595/601H] were stimulated, or not, with R1881 (1 nmol/L) in the presence or absence of GSNO (100 μ mol/L) for 48 hours. ChIP using anti-AR, or control immunoglobulin G (IgG) was performed. Amplified DNA was fractionated on agarose gel and visualized under UV light after staining with ethidium bromide. **B**, PC3 cells were transfected as above and stimulated, or not, with R1881 (1 nmol/L) in the presence or absence of GSNO (100 μ mol/L) for 48 hours. 32 P-labeled oligonucleotides containing FKBP51 promoter sequence were incubated with nuclear extract proteins (5 μ g), fractionated by EMSA on 5% polyacrylamide gel, and visualized by autoradiography. N20, binding mixture contained 0.5 μ g of anti-AR antibody. CR denotes binding samples containing 100-fold excess unlabeled oligonucleotides. All results represent three independent experiments.

for AR binding to AREs. The S-nitrosylation of C601 effectively inhibits AR transcriptional activity, likely as a result of NO destroying zinc-sulfur clusters (44). In the absence of androgen stimulation, AR is expressed in the cytoplasm in complex with HSP90. Available evidence shows that HSP90 binds eNOS and undergoes the S-nitrosylation modification (32), and our results show that SNO-HSP90 transnitrosylates AR. Hence, HSP90 may contribute to maintenance of AR inactivation by a two-step mechanism; sequestration in the cytoplasm and S-nitrosylation. Androgen-induced activation of AR, therefore, may involve AR signal disinhibition as a result of AR dissociation from HSP90 in the cytoplasm, translocation to the nucleus and denitrosylation. Indeed, our preliminary results support the idea that stimulation with androgen provokes a decrease in nuclear AR S-nitrosylation content and a concomitant increase in PSA expression.

The preclinical studies show that systemic treatment of animals harboring xenograft prostate tumors with exogenously supplied NO (i.e., GSNO) inhibits growth of both androgen-dependent and castration-resistant tumors. Remarkably, results of a nonrandomized phase II trial show that treatment of patients who failed primary therapy for clinically localized

prostate cancer with low-dose and sustained delivery of NO-donor nitroglycerin significantly inhibited disease progression as measured with a decrease in PSA doubling time (45), implying that NO abundance inhibits prostate cancer in men. Results of perturbing endogenous levels of NO on the tumor growth rate support the conclusion that NO acts directly on the cancer cells; forced overexpression of eNOS in the cancer cells inhibits tumor growth, whereas knockdown of endogenous eNOS expression accelerates it. However, it remains feasible that the cell-permeable NO impacts other constituents in the tumor mass, such as vascular cells and supporting stroma.

In summary, our results show that increased levels of intracellular NO promotes AR inactivation by S-nitrosylation of a single C601 residue present in the AR DBD and inhibits

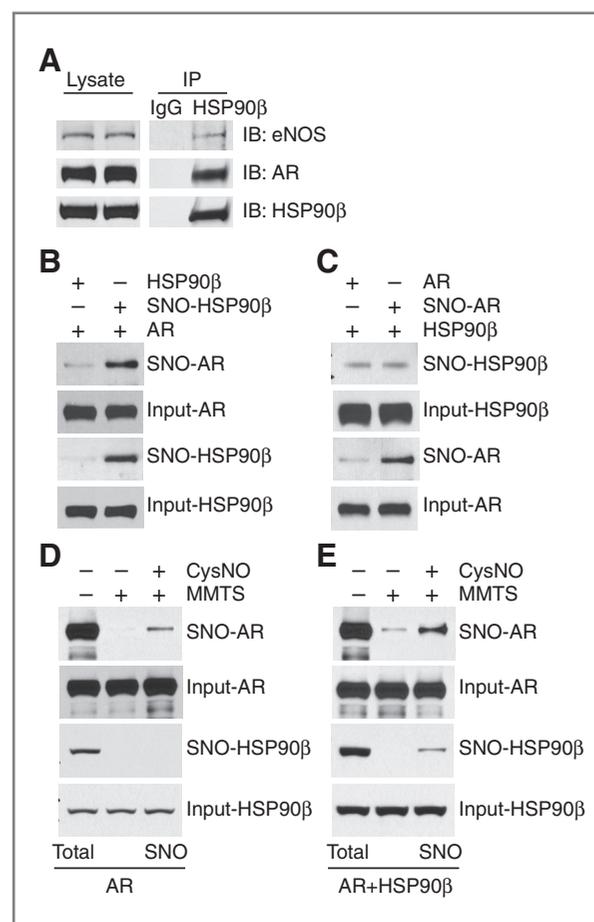


Figure 6. AR is transnitrosylated by HSP90. **A**, HSP90 β forms a complex with AR and eNOS. LNCaP cells were lysed and subjected to immunoprecipitation with anti-HSP90 β or isotype control [immunoglobulin G (IgG)] antibodies. Protein complexes were immunoblotted with the indicated antibodies. **B** and **C**, purified HSP90 β (**B**) or AR (**C**) protein was exposed to CysNO (100 μ mol/L) and resultant S-nitrosylated protein was desalted and then mixed with un-nitrosylated purified protein counterpart. Biotin switch assay was performed to detect the protein transnitrosylation. Input amounts of AR and HSP90 β were verified in each reaction. **D** and **E**, transnitrosylation of AR *in situ*. PC3 cells were transfected with pcDNA3-Flag-AR alone (**D**) or together with pcCMV-Myc-HSP90 β (**E**). Cells were exposed to CysNO (200 μ mol/L), and cell lysates were subjected to the biotin switch assay.

growth of AR-positive androgen-dependent and castration-resistant prostate tumors. In the absence of androgen stimulation, AR is maintained in a transcriptionally inactive state as a result of being sequestered in the cytoplasm and trans-nitrosylated by HSP90. Sequential targeting of individual AR domains with pharmacologically distinct agents may provide a better treatment outcome to more effectively abrogate aberrant and sustained AR signaling in advanced prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Qin, A. Dey, Y. Daaka

Development of methodology: Y. Qin, A. Dey

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Qin, H.T. Purayil

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Qin, A. Dey, Y. Daaka

Writing, review, and/or revision of the manuscript: Y. Qin, A. Dey, Y. Daaka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Qin

Study supervision: Y. Qin, Y. Daaka

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Maintenance of Androgen Receptor Inactivation by S-Nitrosylation

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