

## Small carboxyl-terminal domain phosphatase 2 attenuates androgen-dependent transcription

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**Small carboxyl-terminal domain (CTD) phosphatase 2 (SCP2) was identified and verified as a protein that interacts with the androgen receptor (AR). Ectopic expression of SCP2 or two other family members, SCP1 and SCP3, attenuated AR transcriptional activity in LNCaP cells and were recruited in an androgen- and AR-dependent fashion onto the prostate-specific antigen (PSA) promoter. Silencing SCP2 and SCP1 by short hairpin RNAs increased androgen-dependent transcription of the PSA gene and augmented AR loading onto the PSA promoter and enhancer. SCP2 also attenuated glucocorticoid receptor (GR) function, and its silencing increased dexamethasone-mediated PSA mRNA accumulation and GR loading onto the PSA enhancer in LNCaP 1F5 cells. SCP2 silencing was accompanied by augmented recruitment and earlier cycling of RNA polymerase II on the promoter. Ser 5 phosphorylation of the RNA polymerase II CTD, a process necessary for initiation of transcription elongation, occurred significantly earlier in SCP2-silenced than parental LNCaP cells. Collectively, our results suggest that SCP2 is involved in promoter clearance during steroid-activated transcription.**

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### Introduction

Androgen receptor (AR) mediates the effects of male sex steroids (androgens), testosterone (T), and 5 $\alpha$ -dihydrotestosterone, which are critical in the development and maintenance of the male sexual characteristics. Defective AR function causes several androgen-insensitivity disorders, and aberrant AR signaling is involved in the pathogenesis

of prostate cancer (CaP) (Abate-Shen and Shen, 2000), the most common malignancy among Western men (Parkin *et al*, 2005). AR is a member of the steroid receptor family, a subgroup of the nuclear receptor (NR) superfamily (Beato *et al*, 1995). Like all NRs, AR has a modular structure consisting of a nonconserved amino-terminal domain (NTD), and conserved DNA-binding and carboxyl-terminal ligand-binding domains (Gelman, 2002). Unlike most other NRs, a strong hormone-independent transcription activation function 1 resides within the AR NTD (Rundlett *et al*, 1990; Palvimo *et al*, 1993; Ikonen *et al*, 1997). Upon ligand binding, AR acquires a new conformational state and translocates to the nucleus where it interacts with specific DNA elements, coregulatory proteins (coactivators and corepressors) (McKenna *et al*, 1999; Glass and Rosenfeld, 2000), and the basal transcription machinery (Gelman, 2002; Lee and Chang, 2003). Disturbances in AR functionality caused by altered coregulator interactions/recruitment appear to be linked to the advancement of CaP from a clinically nonaggressive benign form to a metastatic and hormone-refractory form that ultimately becomes untreatable (Abate-Shen and Shen, 2000; Arnold and Isaacs, 2002).

Using a bacteria-based two-hybrid system and a HeLa cell cDNA library, we identified small carboxyl-terminal domain (CTD) phosphatase 2 (SCP2)/OS4 (NM\_005730) (Su *et al*, 1997) as an AR NTD interaction partner. This 32-kDa protein comprises 283 amino acids and shares sequence homology with three other proteins, SCP1 (Yeo *et al*, 2003), SCP3/HYA22 (Ishikawa *et al*, 1997), and the founding member FCP1 (TFIIF-associating CTD phosphatase) (Archambault *et al*, 1997; Cho *et al*, 1999). SCP2 mRNA is expressed in most tissues, including the prostate and testis, and the encoded protein has been implicated in the development of human sarcomas (Su *et al*, 1997). SCP2 and SCP1 have been shown to catalyze the dephosphorylation of both phosphoserine 5 (pS<sup>5</sup>) and phosphoserine 2 (pS<sup>2</sup>) of the RNA polymerase II (Pol II) CTD heptapeptide repeat sequence YSPTSPS (Yeo *et al*, 2003). However, the precise role of SCP2, SCP1, and SCP3 and their involvement in the regulation of CTD phosphorylation/Pol II activity remain unknown (Palancade and Bensaude, 2003; Kashuba *et al*, 2004).

Phosphorylation of the CTD occurs mainly on S<sup>2</sup> and S<sup>5</sup>, although S<sup>7</sup> and Y<sup>1</sup> have also been suggested to be important (Orphanides and Reinberg, 2002; Palancade and Bensaude, 2003; Sims *et al*, 2004). The phosphorylation status of the CTD plays a central role in regulating transcription and ultimately the production of mature mRNA (Orphanides and Reinberg, 2002; Proudfoot *et al*, 2002; Svejstrup, 2004; Zorio and Bentley, 2004). Phosphorylation of S<sup>5</sup> occurs predominantly on the promoter regions in yeast and mammals and is catalyzed by the CDK7 kinase subunit of transcription factor II H (Komarnitsky *et al*, 2000; Cheng and Sharp, 2003; Morris *et al*, 2005). The phosphorylation of S<sup>2</sup> is thought to relieve transcriptional stalling (Lis, 1998) that occurs during elongation and is catalyzed by the CDK9 kinase subunit of

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positive transcription elongation factor b (Shim *et al*, 2002). At the end of the elongation process, pS<sup>2</sup> and pS<sup>5</sup> are dephosphorylated, a step that is required for the reloading of Pol II onto the promoter region (Hausmann and Shuman, 2002; Sims *et al*, 2004).

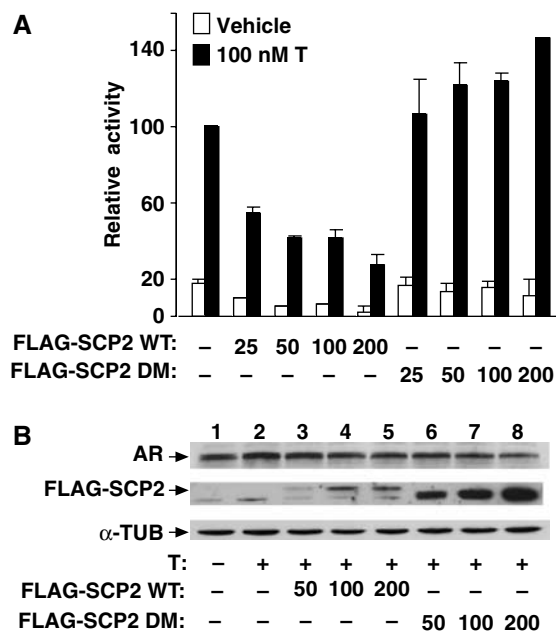
We report here that ectopic expression of SCP2, SCP1, and SCP3 attenuates AR transcriptional activity in LNCaP cells and that SCP2 and SCP1 silencing by lentiviral expression of short hairpin RNAs (shRNA) activates androgen-dependent transcription. Silencing of SCP2 and SCP1 elicits a 2–3-fold increase in the loading of AR onto the prostate-specific antigen (PSA) promoter and enhancer regions. SCP2 silencing increases the amount of Pol II recruited onto the PSA promoter and facilitates the rate of Pol II cycling. In the absence of SCP2, S<sup>5</sup> phosphorylation is achieved more readily, and at steady state, Pol II is loaded onto the PSA promoter at a higher frequency, implying that SCP2 plays a role in regulating AR-dependent transcription. These findings are not AR-specific, as studies carried out with GR suggest that the SCP proteins play a more general role in steroid receptor-mediated signaling.

## Results

Using a HeLa cell cDNA library and the AR NTD as bait, we recovered several positive clones from the bacterial two-hybrid screen. On the basis of DNA sequencing, sequence comparisons, and literature search, nine proteins were considered to be potential AR-interaction partners. The cDNA encoding the carboxyl-terminal sequence of SCP2 (amino acid residues 90–283) was detected twice in the screen. Quantitative real-time PCR (qRT-PCR) analyses of total RNA isolated from HeLa, PC-3, LNCaP, and LNCaP 1F5 cell lines showed that these cell lines had similar SCP2 mRNA levels (not shown). The physical interaction between SCP2 and AR was examined using glutathione S-transferase pull-down assays, mammalian two-hybrid protein–protein interaction assays, and coimmunoprecipitation experiments. These *in vitro* and *in vivo* studies confirmed the bacterial two-hybrid results that SCP2 is capable of direct interaction with AR (Supplementary Figures S1A, B and C).

### SCP2 attenuates AR-dependent transcriptional activation

To study whether SCP2 influences the transactivation function of AR, LNCaP cells were transfected with expression vectors encoding wild-type SCP2 (FLAG-SCP2 WT) or a phosphatase-deficient mutant (FLAG-SCP2 DM) (Kobor *et al*, 1999; Hausmann and Shuman, 2002), together with a PSA promoter-driven luciferase (LUC) reporter, PSA5.8-LUC. Increasing amounts of SCP2 WT brought about a dose-dependent attenuation of AR-dependent transactivation, whereas the phosphatase-deficient SCP2 DM activated AR function to some extent (Figure 1A). The expression of a control reporter, CMV-driven  $\beta$ -galactosidase ( $\beta$ -gal) gene, was not influenced by either SCP2 WT or SCP2 DM, implying that the SCP2 WT-mediated attenuation of AR function was not due to general transcriptional squelching (not shown). There was also an SCP2 dose-dependent decrease of AR activity in vehicle-treated cells. This is due to the residual androgens inherent in our culture medium that elicit a low level activation of AR. Overexpression of either SCP2 WT or



**Figure 1** Overexpression of SCP2 represses AR activity. (A) LNCaP cells were transfected with the indicated amounts (ng) of pFLAG-SCP2 WT or pFLAG-SCP2 DM along with pPSA5.8-LUC (700 ng) and pCMV $\beta$  (200 ng). The total amount of DNA per well was constant. At 24 h after transfection, the cells received either vehicle (open bars) or 100 nM T (closed bars) for the subsequent 24 h. LUC activities were normalized by  $\beta$ -gal activity. The activity of AR alone in the presence of 100 nM T is set as 100. The mean  $\pm$  s.d. values from two independent experiments performed in triplicate are shown. (B) Immunoblot analysis of cell lysates. LNCaP cell lysates from the above experiments were pooled (triplicate wells), resolved by SDS-PAGE, and immunoblotted. AR was probed for using AR3 (upper panel), FLAG-SCP2 WT and DM were probed for using M2 (middle panel), and protein loading was checked with the anti- $\alpha$ -tubulin antibody B-7 (lower panel).

SCP2 DM did not influence AR levels in LNCaP cells (Figure 1B upper panel, lanes 3–8). For the same amount of expression vector, SCP2 DM appeared to be expressed at levels higher than those of SCP2 WT, suggesting that the phosphatase activity plays a role in the turnover of SCP2 protein (Figure 1B middle panel, lanes 3–5 and lanes 6–8). Similar results were obtained with other AR-responsive reporters, including the minimal ARE<sub>2</sub>TATA-LUC reporter (Supplementary Figure S1D). To establish that attenuation of AR function was not due to the mutated receptor present in LNCaP cells (Veldscholte *et al*, 1990), an AR expression vector (pSG5-hAR T877A) (Thompson *et al*, 2003b) carrying the LNCaP mutation was transfected together with PSA5.8-LUC and either SCP2 WT or SCP2 DM into PC-3, HeLa, and COS-1 cells. SCP2 WT attenuated and SCP2 DM increased transactivation function of hAR T877A as efficiently as that of WT AR in all three cell lines (not shown).

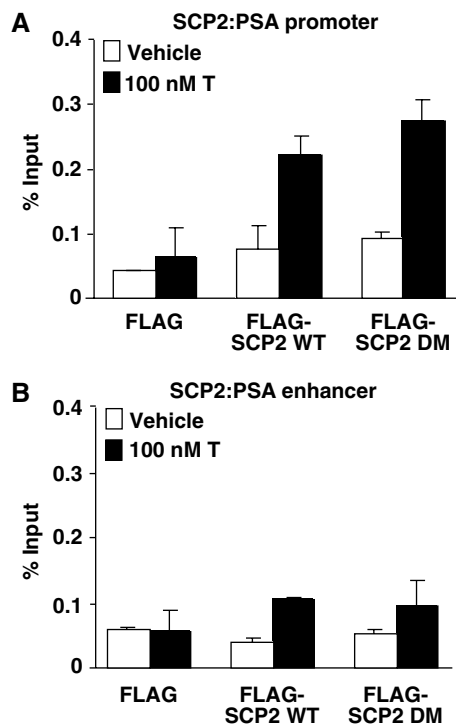
### The influence of SCP1 and SCP3 on AR-dependent transcription

The SCP genes are located on different chromosomes, and expression of all three forms is coordinately regulated in certain cell types (Yeo *et al*, 2005). To investigate whether SCP1 and SCP3 also influence AR activity, LNCaP cells were transfected with increasing amounts of FLAG-SCP1 WT/DM or FLAG-SCP3 WT/DM expression vectors along

with PSA5.8-LUC. Both SCP1 WT and SCP3 WT brought about a dose-dependent attenuation of AR transactivation (Supplementary Figures S2A–D). Once again, the repression was not due to an aberrant expression of AR. The phosphatase-deficient form of SCP1, like SCP2 DM, had a slightly activating effect, but this was not observed with SCP3 DM. Similar results were seen with the minimal ARE<sub>2</sub>TATA-LUC reporter (not shown). These results imply that the SCPs may have overlapping functions *in vivo*.

### SCP2 recruitment onto the PSA promoter and enhancer

To examine whether SCP2 is recruited onto the *PSA* promoter and enhancer, LNCaP cells were transfected with FLAG, FLAG-SCP2 WT or FLAG-SCP2 DM expression vectors and chromatin immunoprecipitation (ChIP) assays were performed with anti-FLAG antibody on cells exposed to T for 2 h. In the absence of T, similar amounts of SCP2 WT and DM were present on the *PSA* promoter and enhancer to those in the FLAG-transfected cells (Figure 2A and B, open bars). Upon T exposure, there was a 2–3-fold increase in the recruitment of SCP2 WT and DM onto the *PSA* promoter (Figure 2A, closed bars), but there was no significant increase in SCP2 on the enhancer (Figure 2B, closed bars). Increased transactivation of AR by SCP2 DM in transient transfection experiments (Figure 1A) is likely to result from the mutant functioning in a dominant negative fashion on the promoter



**Figure 2** Recruitment of SCP2 onto the *PSA* promoter and enhancer. LNCaP cells were transfected with 1  $\mu$ g of FLAG, FLAG-SCP2 WT, or FLAG-SCP2 DM expression vectors and subsequently exposed to vehicle (open bars) or 100 nM T (closed bars) for 2 h before being prepared for ChIP assays. Chromatin samples were immunoprecipitated with M2 and bound DNA was quantified with qRT-PCR. Data presented are normalized to input DNA, and the mean  $\pm$  s.d. values from at least three experiments are shown. (A) FLAG-SCP2 WT or DM levels on the *PSA* promoter; (B) FLAG-SCP2 WT or DM levels on the *PSA* enhancer.

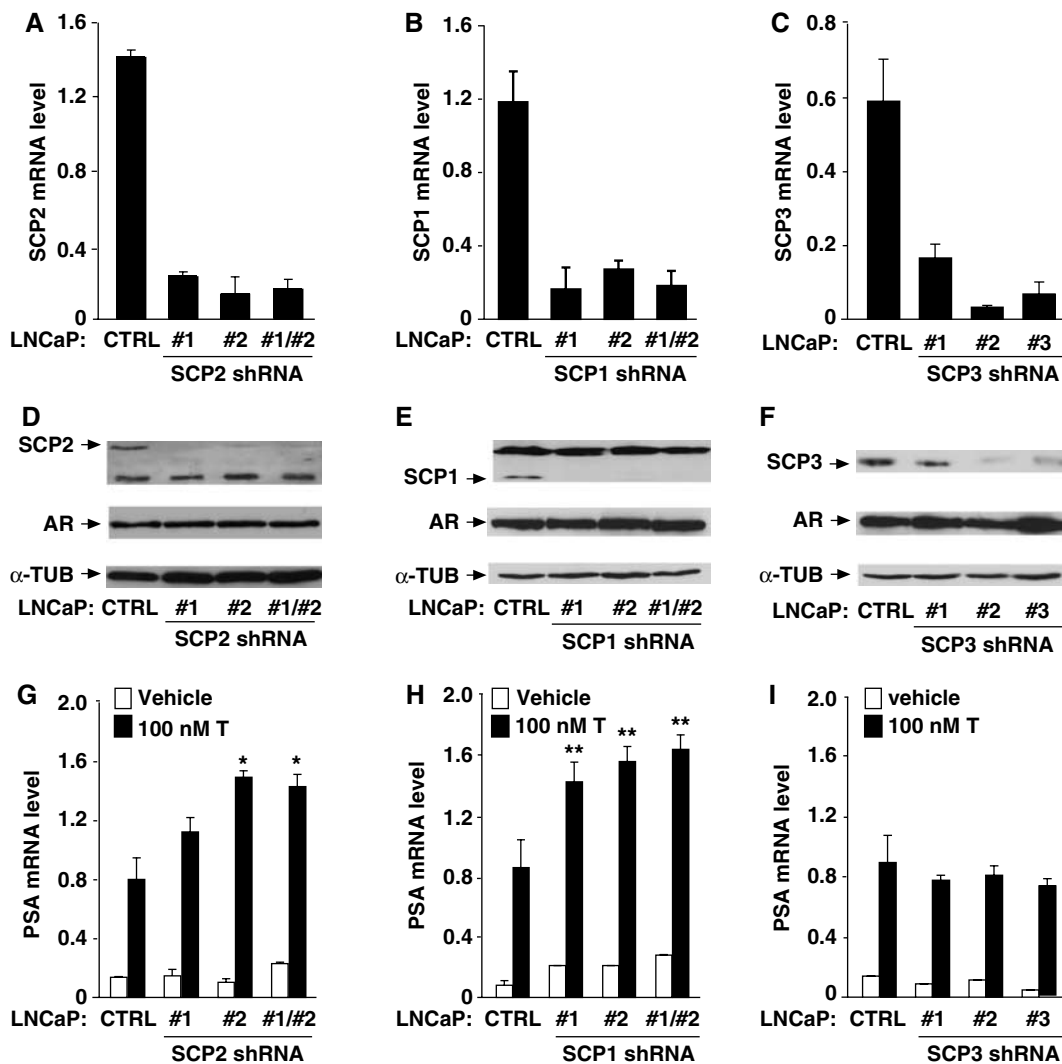
by replacing wild-type SCP from the complex. Under the same experimental conditions, ectopically expressed SCP1 and SCP3 were also recruited in an androgen- and AR-dependent fashion onto the *PSA* promoter but not onto the enhancer (Supplementary Figures S3A and B).

### Silencing of endogenous SCP2, SCP1, and SCP3 in LNCaP cells

To gain more insights into the role of the SCPs on AR function, we designed shRNAs to target and silence each of the three *SCP* genes in LNCaP cells. For each family member, we created three silenced LNCaP cell lines using either single shRNA lentiviral constructs or their combinations (see Supplementary data). An infection rate of >90% was achieved with LNCaP cells, as evidenced by expression of a green fluorescent protein (GFP) encoded by the lentivirus under the control of the CMV promoter. GFP expression remained constant 16 weeks after the infection in SCP2-silenced cells. SCP2, SCP1, and SCP3 mRNA levels in control LNCaP cells (CTRL), expressing empty lentiviral vector, and SCP-silenced LNCaP cells were determined using qRT-PCR at 6, 10, and 120 days (for SCP2) after infection (Figure 3A–C and data not shown). In LNCaP cells, SCP2 shRNA #1 and #2 reduced the level of SCP2 mRNA to 1/15th of that in CTRL cells. In addition, there was a marked reduction in SCP2 protein levels (Figure 3D). SCP1 and SCP3 mRNA levels were not affected by the SCP2 shRNA constructs (not shown). AR protein and mRNA levels in the SCP2-silenced cells were not affected (Figure 3D, Supplementary Figure S4A). Silencing of endogenous SCP2 did not influence cell viability, in that 96 h after the infection, the number of viable cells was essentially the same in CTRL and SCP2-silenced cells (Supplementary Figure S4C). Very similar results were seen in the SCP1- and SCP3-silenced LNCaP cells, except that some residual SCP3 protein was present in the SCP3-silenced cells (Figure 3B, C, E and F, Supplementary Figures S4B and E).

### Influence of SCP silencing on AR-dependent gene expression

To investigate whether SCP silencing influences the expression of AR-regulated genes, we examined the *PSA* gene. SCP2 and SCP1 silencing in LNCaP cells resulted in increased *PSA* mRNA accumulation after 24 h of T exposure by 50–80% (Figure 3G and H). Importantly, re-expression of FLAG-SCP2 WT in SCP2-silenced cells and FLAG-SCP1 WT in SCP1-silenced cells rescued *PSA* mRNA levels back to values similar to those obtained in CTRL cells (Figure 4A and B, gray bars). By contrast, no rescue in *PSA* mRNA levels was seen with the DM forms of SCP2 or SCP1 (Figure 4A and B, open and closed bars). The specificity of these results was assessed by measuring mRNA levels of two androgen-independent transcription factors, TATA-binding protein (TBP) and Sp1. In the presence of T, there were no changes in TBP mRNA levels (Supplementary Figures S4D and E) or Sp1 mRNA levels (not shown) in SCP2- and SCP1-silenced cells, indicating that SCP2 and SCP1 silencing leads to specific consequences in androgen-dependent transcription. Although the SCP3 shRNA constructs elicited a marked depletion in SCP3 mRNA and SCP3 protein levels (Figure 3C and F), they failed to alter *PSA* mRNA accumulation (Figure 3I). A possible explanation for this latter result is that the remaining SCP3 protein content is high enough



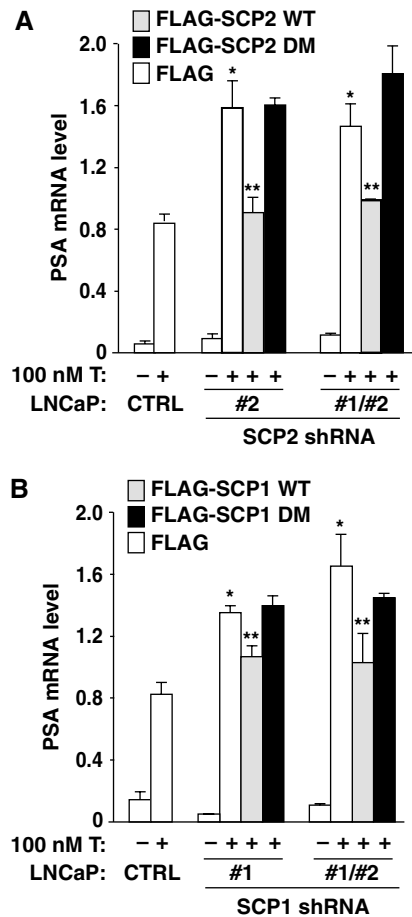
**Figure 3** Silencing of SCP2, SCP1, and SCP3 by shRNA. LNCaP cells were infected with empty lentivirus vector (CTRL) (pLL 3.7) or virus vectors (see Supplementary data for coding sequences) encoding shRNA constructs to target and silence SCP2, SCP1, or SCP3. For each SCP family member, three separate cell lines were produced. (A) SCP2 mRNA 6 days after lentivirus infection with shRNA pLL3.7SCP2 #1, pLL3.7SCP2 #2, or both #1 and #2. (B) SCP1 mRNA 6 days after infection with shRNA SCP1 #1, SCP1 #2, or both #1 and #2. (C) SCP3 mRNA 6 days after infection with shRNA SCP3 #1, SCP3 #2, or SCP3 #3. SCP mRNA levels were measured with qRT-PCR using primers specific for SCP2, SCP1, and SCP3, respectively. All results were normalized to  $\beta$ -actin mRNA levels, and the mean  $\pm$  s.d. values from two independent experiments are shown. (D–F) Immunoblot analysis of SCP and AR protein levels 6 days after lentiviral infection. Protein samples from whole-cell lysates were separated on 12% SDS–PAGE gels. The expressions of the SCP family members (150  $\mu$ g protein/lane) were probed with 6703 antiserum (D–F upper panels). AR expression of the different cell lines (50  $\mu$ g protein/lane) was probed with AR3 (middle panels). Protein loading of the samples was checked by reprobing the AR membrane with B-7 (lower panels). (G–I) PSA mRNA levels in CTRL, SCP2-, SCP1-, and SCP3-silenced LNCaP cells. Cells were cultured in hormone-depleted medium for 4 days and received thereafter vehicle (open bars) or 100 nM T (closed bars) for the subsequent 24 h. Cells were harvested for qRT-PCR. \* $P$ <0.05; \*\* $P$ <0.01 in comparison to CTRL cells.

to prevent an increase in PSA mRNA accumulation from occurring.

#### AR loading onto the PSA promoter and enhancer

We used ChIP assays to examine whether the increases in PSA mRNA accumulation in SCP2- and SCP1-silenced cells were accompanied by changes in AR loading onto the PSA promoter and enhancer. CTRL, SCP2-, and SCP1-silenced (shRNA SCP2 #1 plus #2- and shRNA SCP1 #1-expressing cell lines) LNCaP cells were treated with T and collected at timed intervals over an 8-h period, after which chromatin samples were immunoprecipitated with an AR antiserum and bound DNA was quantified. At time point 0 (min), the loading of AR onto both the PSA promoter and enhancer was

indistinguishable between CTRL and SCP2-silenced cells. In agreement with our previous results (Kang *et al*, 2004), we observed cyclic loading and unloading of AR onto the promoter and enhancer, with some 10 times more AR being loaded onto the enhancer than the promoter, and found no change in the rate at which this happened in the silenced cells (Figure 5A and B). After 30 min of T treatment, there was almost twice as much AR loaded onto the promoter region in the SCP2-silenced than in the CTRL cells. AR loading onto the promoter region was maximal at 150 and 240 min in the silenced and CTRL cells, but there was 3–4 times more AR on the promoter in the silenced cells at the time points. Maximal AR loading onto the enhancer in both the silenced and CTRL cells was seen at 120 min, and at this time point, there was

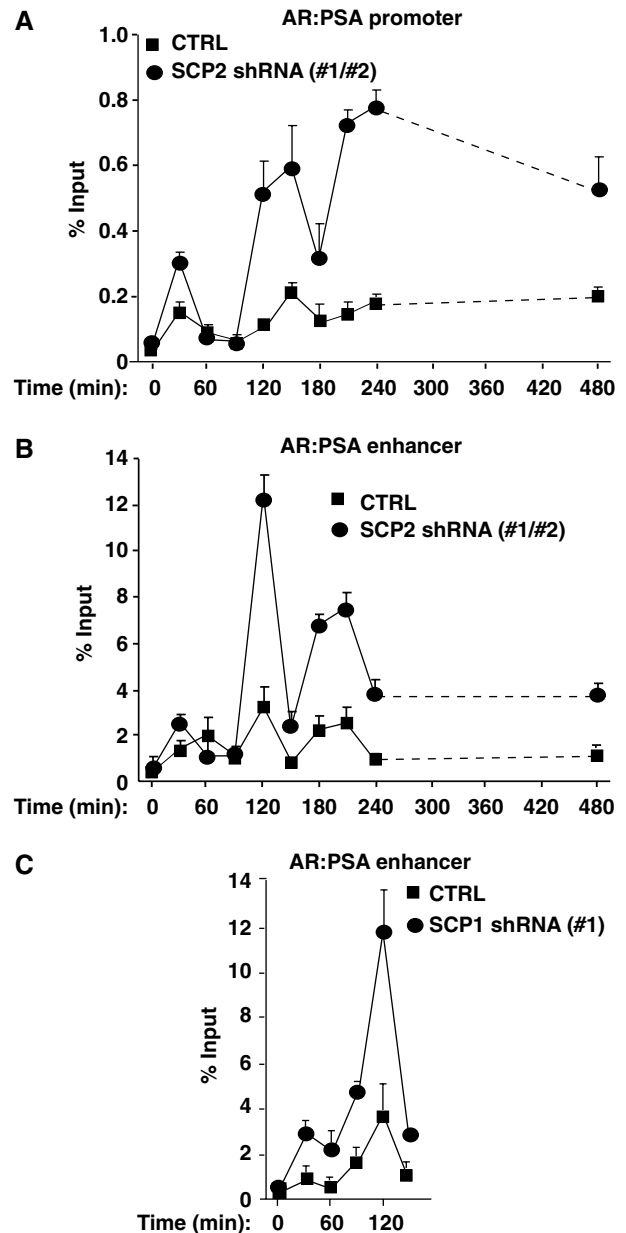


**Figure 4** Re-expression of SCP2 or SCP1 rescues the silencing effects. (A) pFLAG (200 ng), pFLAG-SCP2 WT (200 ng), or pFLAG-SCP2 DM (200 ng) were transfected into SCP2-silenced cells and cultured/processed as described in the legend to Figure 3G and H. (B) Essentially the same experiment as in (A) except that FLAG-SCP1 WT/DM were transfected into SCP1-silenced cells. \* $P < 0.05$ , CTRL versus SCP2 shRNA; \*\* $P < 0.05$ , silenced versus rescued cells.

again 3–4 times more AR loaded in the silenced cells; this amount remained elevated over CTRL cells throughout the time course. Similar results were obtained with SCP1-silenced cells (Figure 5C and data not shown), suggesting that these two SCP proteins have redundant functions in the regulation of AR promoter and enhancer loading.

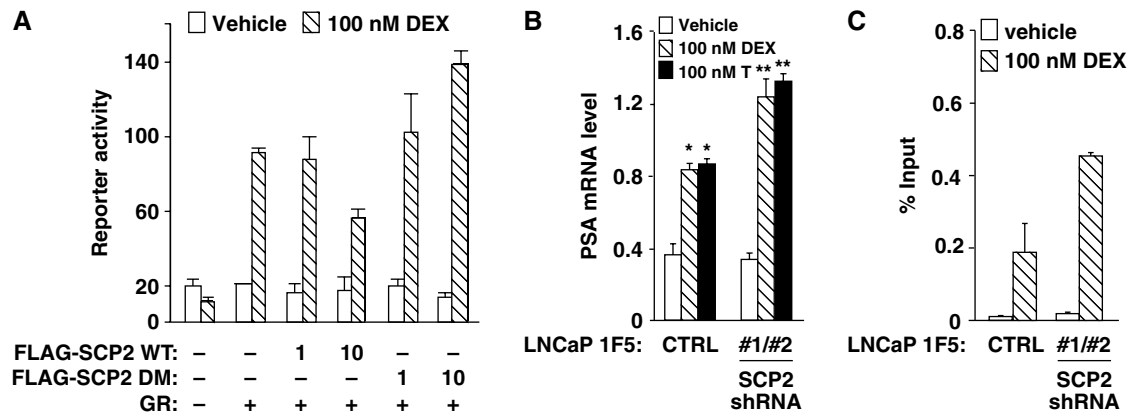
**SCP2 influences the function of other steroid receptors**

The phenomena described above are not specific for AR, as overexpression of SCP2 WT also repressed transcriptional activity of glucocorticoid (GR) (Figure 6A), estrogen, and progesterone receptors (not shown). To investigate whether endogenous SCP2 influences GR-dependent gene expression, we silenced SCP2 by shRNA expression in LNCaP 1F5 cells (Supplementary Figures S5A, B and C). Parental LNCaP cells do not express GR; however, LNCaP 1F5 cells have been engineered to express rat GR in a stable fashion and shown to exhibit increased PSA mRNA accumulation in response to dexamethasone (DEX) (Cleutjens *et al*, 1997). In agreement with previous results, 24-h DEX exposure elicited a clear increase in PSA mRNA accumulation that was further augmented by ~50% in the SCP2-depleted LNCaP 1F5 cells (Figure 6B, hatched bars). In agreement with Cleutjens *et al*



**Figure 5** AR loading onto the PSA promoter and enhancer. CTRL, SCP2-, and SCP1-silenced LNCaP cell lines were treated with 100 nM T and harvested for ChIP assays at 30 min intervals over an 8-h period. Chromatin samples were immunoprecipitated with AR3 and bound DNA was quantified with qRT-PCR. Data presented are normalized to input DNA, and the mean  $\pm$  s.d. values from at least three experiments is shown. (A) AR loading onto the PSA promoter in SCP2-silenced cells; (B) AR loading onto the PSA enhancer in SCP2-silenced cells; (C) AR loading onto the PSA enhancer in SCP1-silenced cells.

(1997), T response was similar to that of DEX in LNCaP 1F5 cells (Figure 6B, solid bars). In view of the fact that DEX regulates PSA mRNA accumulation, it was pertinent to examine DEX-dependent GR loading onto the promoter and enhancer regions of the PSA gene in these cells. CTRL and SCP2-silenced LNCaP 1F5 cells treated with DEX for 2 h were subjected to ChIP assays with a GR antibody, and the results showed that GR is indeed loaded in a DEX-dependent fashion onto the PSA promoter and enhancer (Figure 6C and data not shown). Importantly, similar to AR loading (Figure 5), DEX



**Figure 6** Effects of SCP2 on GR-dependent transcription. (A) Overexpression of SCP2 attenuates GR-mediated transcription. COS-1 cells were transfected with the indicated amounts (ng) of FLAG-SCP2 WT or FLAG-SCP2 DM along with pSG5-hGR (2 ng), pARE<sub>4</sub>tk-LUC (200 ng), and  $\beta$ -gal (50 ng). The total amount of DNA per well was constant. At 24 h after transfection, the cells received either vehicle (open bars) or 100 nM DEX (hatched bars) for the subsequent 24 h. LUC activities were normalized by  $\beta$ -gal activity. The mean  $\pm$  s.d. values from an experiment performed in triplicate are shown. (B) PSA mRNA levels in CTRL and SCP2-silenced LNCaP 1F5 cells. Essentially the same experiment as in Figure 3G, except that LNCaP 1F5 cells were exposed to vehicle (open bars), 100 nM DEX (hatched bars), or 100 nM T (closed bars) for 24 h. \* $P < 0.01$ , vehicle versus DEX or T treatment; \*\* $P < 0.01$ , DEX or T treatment in CTRL versus SCP2-silenced cells. (C) GR loading onto the PSA enhancer region in CTRL and SCP2-silenced LNCaP 1F5 cells. Essentially the same experiment in Figure 5, except that CTRL and SCP2-silenced LNCaP 1F5 were exposed to vehicle (open bars) or 100 nM DEX (hatched bars) for 2 h and chromatin samples were immunoprecipitated with M-20.

loading onto the PSA enhancer was over two-fold higher in the SCP2-depleted than in CTRL LNCaP 1F5 cells (Figure 6C). Collectively, these data imply that SCP2 influences transcriptional mechanisms that are not unique to AR, but are shared by other steroid receptors as well.

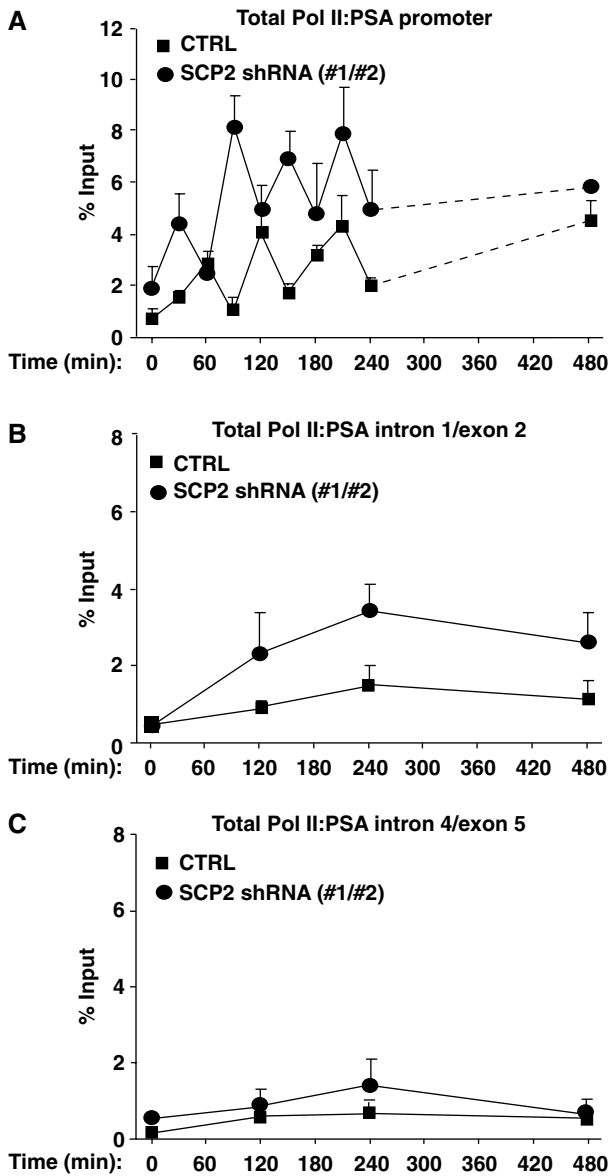
#### Pol II, pS<sup>5</sup>, and pS<sup>2</sup> levels on the PSA gene

Even though the SCP orthologs may have redundant functions, our subsequent experiments were conducted only with SCP2. We next examined whether increased AR loading onto the PSA promoter and enhancer in the SCP2-silenced LNCaP cells influenced the recruitment of Pol II onto the PSA promoter and the extent of CTD phosphorylation, that is, the levels of pS<sup>2</sup> and pS<sup>5</sup>. We first validated total Pol II, pS<sup>5</sup>, and pS<sup>2</sup> antibodies by immunoblotting whole-cell extracts from CTRL and SCP2-silenced LNCaP cells in the absence and presence of T. There was no difference between CTRL and silenced cells treated with or without T in the amount of Pol II (Supplementary Figure S6, upper panel), pS<sup>5</sup> (middle panel), and pS<sup>2</sup> (lower panel). Thus, any difference detected by the ChIP assays should reflect an androgen-dependent phenomenon.

We followed total Pol II recruitment onto the promoter of the PSA gene over an 8-h time period of T treatment in CTRL and SCP2-silenced LNCaP cells (Figure 7A). At time point 0 (min), Pol II loading onto the PSA promoter in SCP2-silenced cells (shRNA #1 plus #2-expressing cell line) was twice as much as that in CTRL cells. Pol II recruitment demonstrated cyclicity in both CTRL and SCP2-silenced cells. However, the peaks of Pol II recruitment were achieved earlier in SCP2-silenced cells. Pol II recruitment onto the PSA promoter was increased in SCP2-silenced cells over that in CTRL cells throughout the time course. We also investigated Pol II levels within the intragenic regions of the PSA gene. Using primers directed towards intron 1/exon 2 and intron 4/exon 5 (exon 5 being the last exon of the PSA gene) regions of the PSA gene, we tracked the levels of Pol II over the 8-h period (Figure 7B

and C). As previously reported (Lis, 1998; Cheng and Sharp, 2003; Morris *et al*, 2005), there was a slight drop in Pol II levels already within the intron 1/exon 2 region as compared to the promoter region (cf. % input values in Figure 7, panels A and B); however, Pol II levels in the silenced cells were elevated over CTRL during the entire time course, suggesting that in the absence of SCP2, Pol II is cleared from the promoter region onto the PSA gene at an increased rate compared to CTRL cells. We observed further decreases in Pol II within the intron 4/exon 5 region (cf. % input values in Figure 7, panels A and C), and both silenced and CTRL cells had a similar amount of Pol II. The reduction and convergence in the amount of total Pol II between the silenced and CTRL cells is not necessarily a PSA-specific process; it may be due, in part, to polymerase stalling, active gene dissociation, or it may reflect a process involved with elongation termination (Mason and Struhl, 2005; Morris *et al*, 2005).

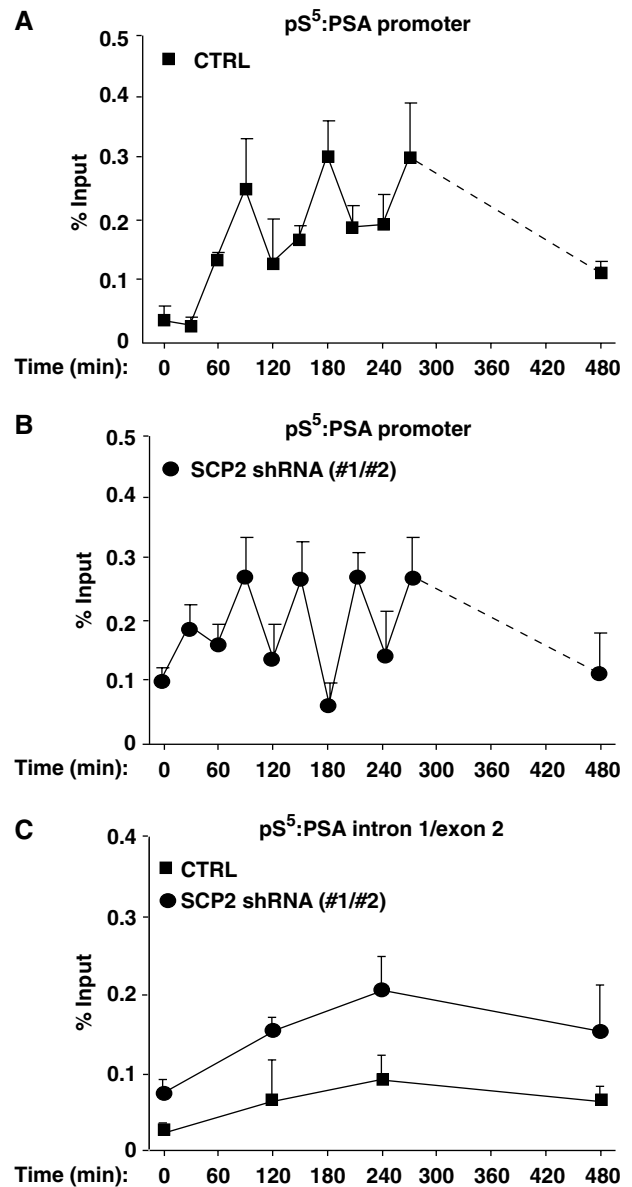
It has been shown in yeast (Komarnitsky *et al*, 2000), and recently in human cells (Morris *et al*, 2005), that during the residency of Pol II on the promoter, the CTD is preferentially phosphorylated on S<sup>5</sup> and the phosphorylation status of S<sup>5</sup> is a rate-limiting step for promoter clearance and initiation of the elongation phase in the Pol II transcriptional cycle. Using phosphopeptides, it has been reported that SCP2 preferentially dephosphorylates pS<sup>5</sup> of the Pol II CTD (Yeo *et al*, 2003). We used ChIP assays with antibodies directed against pS<sup>5</sup> and pS<sup>2</sup> to examine the phosphorylation of these two Ser residues on the PSA promoter upon T exposure. Phosphorylation of S<sup>5</sup> occurred in a cyclic fashion on the promoter (Figure 8A and B). At time point 0, the amount of pS<sup>5</sup> on the PSA promoter was two-fold higher in the silenced cells than the CTRL cells. In addition, pS<sup>5</sup> levels increased after 30 min of T exposure in the silenced cells, whereas this increase commenced only after 60 min in CTRL cells. Interestingly, maximal pS<sup>5</sup> levels were similar in both cell lines; however, S<sup>5</sup> phosphorylation occurred more rapidly in the silenced cells. pS<sup>5</sup> levels within the intron 1/exon 2 region were elevated in the silenced cells



**Figure 7** Total Pol II on the *PSA* gene. CTRL and SCP2-silenced LNCaP cells were cultured and prepared as described in the legend to Figure 5. (A) Total Pol II recruitment onto the *PSA* promoter; (B) total Pol II within the *PSA* intron 1/exon 2 region; and (C) total Pol II within the *PSA* intron 4/exon 5 region.

over CTRL cells (Figure 8C). The increased amount of pS<sup>5</sup> in the silenced cells was in proportion to that of total Pol II in this region. Therefore, the elevated pS<sup>5</sup> in the silenced cells may be due to increased amount of Pol II undergoing promoter clearance, rather than being a specific SCP2-silencing effect. pS<sup>5</sup> levels within the intron 4/exon 5 region were reduced to a similar level in both cell lines (not shown), reflecting the situation with total Pol II.

We next examined pS<sup>2</sup> levels over the *PSA* gene. Phosphorylation of S<sup>2</sup> occurs later during the elongation phase, and in agreement with previous results (Morris *et al*, 2005), we saw very little, if any, pS<sup>2</sup> on the *PSA* promoter (Figure 9A). We observed a small increase in pS<sup>2</sup> levels within the intron 1/exon 2 region after T treatment in both CTRL and SCP2-silenced cells (Figure 9B). More marked increases in pS<sup>2</sup> levels were seen within the intron 4/exon 5

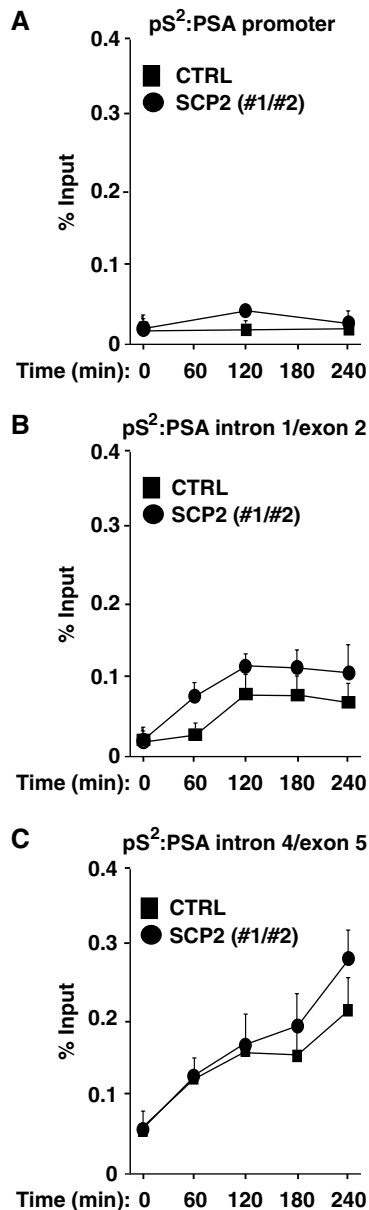


**Figure 8** pS<sup>5</sup> levels on the *PSA* gene. CTRL and SCP2-silenced cells were cultured and prepared as described in the legend to Figure 5. (A) pS<sup>5</sup> levels on the *PSA* promoter in CTRL cells; (B) pS<sup>5</sup> levels on the *PSA* enhancer in SCP2-silenced cells; and (C) pS<sup>5</sup> levels within the intron 1/exon 2 region in CTRL and SCP2-silenced cells.

region, but again, there were no differences between the cell types (Figure 9C). These results suggest that SCP2 does not influence the phosphorylation status of S<sup>2</sup> either on the promoter or in intragenic regions of the *PSA* gene.

## Discussion

AR function regulates the effects of androgens that are critical in the development and maintenance of the male phenotype. Disruptions in androgen signaling generally derive from one of the three reasons; aberrant androgen binding, impaired DNA binding, or altered coregulator interaction (Gelmann, 2002). Altered coregulator interaction and/or recruitment appear to be linked to the pathogenesis of CaP from treatable primary hormone-responsive CaP to untreatable and lethal hormone refractory CaP, a process called 'androgen escape'



**Figure 9** pS<sup>2</sup> levels on the *PSA* gene. CTRL and SCP2-silenced cells were cultured and prepared as described in the legend to Figure 5. (A) pS<sup>2</sup> levels on the *PSA* promoter in CTRL and SCP2-silenced cells; (B) pS<sup>2</sup> levels within the intron 1/exon 2 region in CTRL and SCP2-silenced cells; and (C) pS<sup>2</sup> levels within the intron 4/exon 5 region in CTRL and SCP2-silenced cells.

(Abate-Shen and Shen, 2000; Arnold and Isaacs, 2002; Edwards and Bartlett, 2005). Very little is known about the molecular mechanisms behind androgen escape; however, it has been linked to AR mutations that disrupt interactions with coregulatory proteins (Glass and Rosenfeld, 2000; Thompson *et al*, 2003a; Linja *et al*, 2004). In this work, we searched for novel AR coregulatory proteins that interact with the AR NTD and identified SCP2, a protein phosphatase previously implicated in transcriptional regulation by GR and thyroid hormone receptors (Yeo *et al*, 2003).

SCP2 is a member of the newly identified class C protein phosphatases that includes SCP1, SCP3, and FCP1. All family members share the same catalytic motif  $\psi\psi\psi\psi\text{DXDX}(\text{T/V})\psi\psi$  (where  $\psi$  is a hydrophobic residue) (Das *et al*, 1996; Barford

*et al*, 1998), and both D residues are required for catalytic phosphatase activity (Collet *et al*, 1998; Hausmann and Shuman, 2002). The family members are encoded by distinct genes located on different chromosomes, and SCP1, SCP2, and SCP3 are very similar in size and amino-acid sequence (Yeo *et al*, 2003; Kamenski *et al*, 2004). FCP1 is conserved throughout the eukaryotes, is essential for cell survival, and binds S<sup>2</sup> and S<sup>5</sup> phosphorylated Pol II (Kobor *et al*, 1999; Lin *et al*, 2002; Yu *et al*, 2003). All members of this phosphatase family are implicated in CTD dephosphorylation (Yeo *et al*, 2003; Palancade *et al*, 2004); however, the ways by which specificity between the members and mechanism of action are achieved are still unknown (Barford *et al*, 1998; Palancade and Bensaude, 2003; Meinhart *et al*, 2005).

The phosphorylation status of the Pol II CTD plays a central role in regulating transcription and production of mature mRNA (Orphanides and Reinberg, 2002; Proudfoot *et al*, 2002; Zorio and Bentley, 2004). The crystal structure of Pol II has revealed that the CTD, containing up to 52 repeats of the heptapeptide sequence in mammals (Cramer *et al*, 2001), is responsible for directing mRNA-processing events such as 5'-capping, intron splicing, and 3'-end formation (Bird *et al*, 2004; Zorio and Bentley, 2004). The transcription cycle has five main phases: preinitiation, initiation, promoter clearance, elongation, and termination. Transcription preinitiation/Pol II promoter loading requires the CTD to be unphosphorylated; however, promoter clearance and transcription elongation necessitates the CTD to be hyperphosphorylated that enables the CTD to recruit the auxiliary proteins required for mRNA processing (Palancade *et al*, 2004; Meinhart *et al*, 2005). At the end of transcription elongation, the CTD has to return to the dephosphorylated state for the preinitiation phase to begin again (Hirose and Manley, 2000; Komarnitsky *et al*, 2000). S<sup>5</sup> phosphorylation plays an important part in transcription initiation/promoter clearance and recruitment of the 5' mRNA-capping machinery (Komarnitsky *et al*, 2000; Morris *et al*, 2005). In mammals and *Drosophila*, 5'-mRNA capping may be connected to proximal promoter pausing of Pol II, a phenomenon where transcription elongation stalls (Lis, 1998), which has been shown to be relieved by S<sup>2</sup> phosphorylation (Shim *et al*, 2002), enabling elongation to proceed to the termination step, where S<sup>2</sup> and S<sup>5</sup> are dephosphorylated by FCP1 (Kobor *et al*, 1999). After CTD dephosphorylation, Pol II can be recycled back onto the promoter for preinitiation (Hausmann and Shuman, 2002; Sims *et al*, 2004).

In this work, we have shown that the SCPs have several properties that influence AR-dependent regulation of transcription and that other steroid receptors may be affected in a comparable fashion. First, in the absence of SCP2 or SCP1, more AR is loaded onto the *PSA* promoter and enhancer upon T treatment. DEX-dependent loading of GR on the *PSA* regulatory regions is influenced in a similar manner. Second, more Pol II is recruited to the promoter region, and third, phosphorylation of S<sup>5</sup> is achieved earlier and more rapidly, resulting in an increase in *PSA* mRNA accumulation within 24 h. The activity of SCP2 and SCP1 is stimulated by the RAP74 subunit of transcription factor IIF (TFIIF) (RAP30/RAP74) (Yeo *et al*, 2003). TFIIF has been shown to be recruited by the AR NTD (McEwan and Gustafsson, 1997; Reid *et al*, 2002) to form part of the preinitiation complex (Lee and Chang, 2003) that is required for AR-directed Pol II

transcription initiation (Reines *et al*, 1996; Dvir *et al*, 2001). Therefore, the SCPs seem to exhibit a rate-controlling function in the feed forward cycle that we found to occur in the absence of SCP2; increased AR recruitment, increased Pol II recruitment/promoter loading, quicker S<sup>5</sup> phosphorylation, and higher rate of transcription. In the absence of SCP2, increased androgen-dependent loading of AR (or glucocorticoid-dependent loading of GR) and recruitment of Pol II onto the *PSA* promoter may be owing to expedited Pol II promoter clearance caused by an accelerated rate of S<sup>5</sup> phosphorylation. The higher rate of promoter clearance may require more AR (or GR) and unphosphorylated Pol II to be recruited onto the promoter to keep-up with the increased rate of transcription. Our data also suggest that SCP2 mainly influences Pol II phosphorylation within the promoter region of the *PSA* gene and that dephosphorylation of pS<sup>5</sup> occurs at the end of the elongation phase rather than being replaced by pS<sup>2</sup> during elongation initiation. Unlike FCP1, which in yeast associates with phosphorylated Pol II during the elongation phase (Kong *et al*, 2005), our results indicate that SCP2 influences Pol II phosphorylation levels only within the promoter region.

Collectively, our results suggest that the SCPs are key components in the regulation of androgen-dependent transcription. Although most of the experiments in this work were carried out with AR and SCP2, the studies performed with the other SCP proteins and GR imply that the SCPs may have a more general role in steroid receptor-dependent signaling. It is tempting to suggest that recruitment of SCP proteins by steroid receptors onto gene regulatory regions is one of the mechanisms by which the receptors control the rate of promoter clearance as well as the cyclicity in receptor loading.

## Materials and methods

### Plasmids

Bacterial two-hybrid system plasmids were purchased (Stratagene). The NTD of human AR (amino acids 1–556) was fused in-frame to bacteriophage  $\lambda$  repressor protein ( $\lambda$ cI) to create pBT-hAR NTD 1–556 using PCR as described in Supplementary data. Mammalian two-hybrid system plasmids were purchased (BD Biosciences Clontech). pVP16-hAR NTD (amino acids 1–556) was created by PCR techniques (see Supplementary data). SCP2 expression vector was purchased (Invitrogen) and used to create a Gal4 DBD fusion of SCP2 and pFLAG-CMV2 (Sigma-Aldrich). SCP1 and SCP3 expression vectors were purchased (Origene). To express soluble SCP2, a truncated SCP2, pGEX-6P-1 SCP2T (residues 90–283), was created (see Supplementary data). The phosphatase-inactive SCP2 (SCP2 DM) was created by incorporating the double mutation to D96E and D98N (Kobor *et al*, 1999; Hausmann and Shuman, 2002) into the active site (VVID<sup>96</sup>LD<sup>98</sup>ETLV) of SCP2, as described in Supplementary data. pCMV-hAR, pSG5-hAR and pSG5-hGR have been described (Simental *et al*, 1991; Adeyemo *et al*, 1993; Thompson *et al*, 2003b). pPSA5.8-LUC contains proximal promoter with two AREs (ARE I at nt –170 and ARE II at nt –394) and enhancer (ARE III) at nt –4200 driving the LUC gene. pG5-LUC was purchased (Promega). pCMV $\beta$  was used to assess transfection efficiency (BD Biosciences Clontech). The lentiviral transfer vector pLentiLox3.7 mU6 (pLL3.7) (Rubinson *et al*, 2003) containing a mouse U6 RNA pol III promoter was a gift from Dr Van Parijs (MIT, Boston). The packaging vectors were provided by the Biomedicum Helsinki Virus Core Facility. shRNA sequences are available in Supplementary data.

### Antibodies

Anti-SCP antiserum (6703) that recognizes all three SCP proteins was a gift from Dr Samuel Pfaff (The Salk Institute, La Jolla, CA). Anti-AR antiserum (AR3) was raised against full-length rat AR (see

Supplementary data). Anti-GR Ab (M-20) and anti-Pol II Ab (N-20) were purchased (Santa Cruz Biotechnology Inc.). The mouse monoclonal antibodies used were anti-Pol II pS<sup>2</sup> (H5) and anti-Pol II pS<sup>5</sup> (H14) (Covance); anti-VP16 (14-5) and anti- $\alpha$ -tubulin (B-7) (Santa Cruz Biotechnology Inc); and anti-FLAG (M2) (Sigma-Aldrich).

### Bacterial two-hybrid screen

The screen was performed according to the manufacturer's instructions (Stratagene) using pBT-hAR NTD 1–556 as bait and a HeLa cDNA library (Stratagene). Recovered cDNA sequences were subjected to a BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) search. For detailed description, see Supplementary data.

### Cell culture and transfection

HeLa, PC-3, and COS-1 cells (all from ATCC) were maintained and transfected as described (Poukka *et al*, 2000; Thompson *et al*, 2001). LNCaP (ATCC) and LNCaP 1F5 cells, a gift from Dr Jan Trapman (Erasmus University Medical Center, The Netherlands), were maintained and transfected as described in Supplementary data.

### Purification of GST proteins and GST pull-down assays

Production and purification of GST fusion proteins was performed essentially as described (Kotaja *et al*, 2002). BL21 CodonPlus bacteria transformed with GST or GST-SCP2T expression vectors were treated with 0.2 mM IPTG and incubated at 20°C for 15 h (see Supplementary data).

### Coimmunoprecipitation and immunoblotting

COS-1 cells were transfected with expression vectors encoding FLAG-SCP2 WT and AR essentially as described (Thompson *et al*, 2001) and immunoprecipitated using the anti-FLAG antibody and GammaBind G Sepharose for 6 h at 4°C (see Supplementary data). Immunocomplexes were visualized using the ECL Western blot reagents (GE Healthcare). Immunoblot analysis of whole-cell extracts and transfected cells were prepared and probed as described (Thompson *et al*, 2001).

### Lentivirus generation and transduction

Lentivirus production was performed as described in Supplementary data.

### qRT-PCR

All samples and reagents were prepared or purchased for qRT-PCR using the LightCycler and SYBR green I dye system (Roche Molecular Systems). Primer sequences have been published: AR and  $\beta$ -actin (Hirvonen-Santti *et al*, 2003); rat GR (Samtani *et al*, 2006); SCP1, SCP2, and SCP3 (Wang and Seed, 2003); PSA (Kang *et al*, 2004); and TBP (Linja *et al*, 2001). Results were normalized to  $\beta$ -actin values of the respective sample. Additional details are given in Supplementary data.

### Flow cytometry analysis

Enhanced GFP expression in lentivirus-infected LNCaP cells was analyzed by fluorescence-assisted cell sorting (FACS). Background fluorescence was set using noninfected LNCaP cells. Percentages of GFP-positive cells in shRNA-expressing LNCaP cells were estimated using CellQuest software.

### Cell viability assay

A colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 (Roche Molecular Systems) by mitochondrial dehydrogenases, was used to quantify cell viability and proliferation. The assays were performed according to the manufacturer's instructions.

### ChIP

ChIP assays were performed essentially as previously described (Kang *et al*, 2002). The TaqMan assays for the quantification of the genomic intron1/exon 2 and intron 4/exon 5 *PSA* sequences were designed by TIB MOLBIOL, sequences in Supplementary data.

### Statistical analyses

Statistical analysis of the results was performed by Student's *t*-test.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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