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The E3 Ubiquitin Ligase Siah2 Contributes to Castration-Resistant Prostate Cancer by Regulation of Androgen Receptor Transcriptional Activity

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SUMMARY

Understanding the mechanism underlying the regulation of the androgen receptor (AR), a central player in the development of castration-resistant prostate cancer (CRPC), holds promise for overcoming the challenge of treating CRPC. We demonstrate that the ubiquitin ligase Siah2 targets a select pool of NCOR1-bound, transcriptionally-inactive AR for ubiquitin-dependent degradation, thereby promoting expression of select AR target genes implicated in lipid metabolism, cell motility, and proliferation. Siah2 is required for prostate cancer cell growth under androgen-deprivation conditions in vitro and in vivo, and Siah2 inhibition promotes prostate cancer regression upon castration. Notably, Siah2 expression is markedly increased in human CRPCs. Collectively, we find that selective regulation of AR transcriptional activity by the ubiquitin ligase Siah2 is important for CRPC development.

INTRODUCTION

In American men, prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death. Signaling through the androgen receptor (AR), a member of the nuclear receptor superfamily activated by steroids, plays an essential role in the initiation and progression of PCa (Shen and Abate-Shen, 2010). AR consists of an N-terminal domain, a central DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD). AR transcriptional activity is mediated via AF1 and AF2, two transactivation domains located within the N-terminal and the LBD domains, respectively. Upon ligand binding, AR translocates to the nucleus and regulates gene expression through binding to androgen-responsive elements (AREs) on the AR target genes.

Given the central role AR plays in the development of PCa, androgen-deprivation therapy (ADT) is used as a first-line treatment for metastatic PCa. Although such therapy achieves significant clinical response, patients with advanced prostate cancer invariably relapse with a more aggressive form of PCa known as castration-resistant PCa (CRPC). Studies on the pathogenesis of CRPC have revealed that resumption of AR-dependent transcriptional activity is a critical event in nearly all cases (Waltering et al., 2012). Several mechanisms have been suggested to mediate AR reactivation during CRPC progression, including AR gene amplification or overexpression, AR mutations conferring ligand promiscuity, expression of AR splice variants allowing androgen-independent activity, and intratumoral androgen production.

Similar to other transcription factors, AR is subject to regulation by the ubiquitin-proteasome pathway, and the E3 ubiquitin ligases Mdm2 and CHIP have been implicated in the control of AR stability and activity (Chymkowitch et al., 2011; Lin et al., 2002). In humans, Siah1 and Siah2 comprise a two-member

Significance

Increased androgen receptor (AR) activity is central to the development of castration-resistant prostate cancer (CRPC), which is a major obstacle to the treatment of advanced/metastatic prostate cancer with hormone therapy. We find that the ubiquitin ligase Siah2 enhances the transcriptional activity of AR by degrading a transcriptionally-inactive pool of AR on select gene promoters/enhancers. Consequently, Siah2 promotes the expression of select AR target genes, leading to the growth of CRPC cells under androgen-deprivation conditions. Our findings point to the importance of targeting a select subpopulation of AR target genes for the treatment of CRPC and the possible consideration of Siah2 for such an approach.

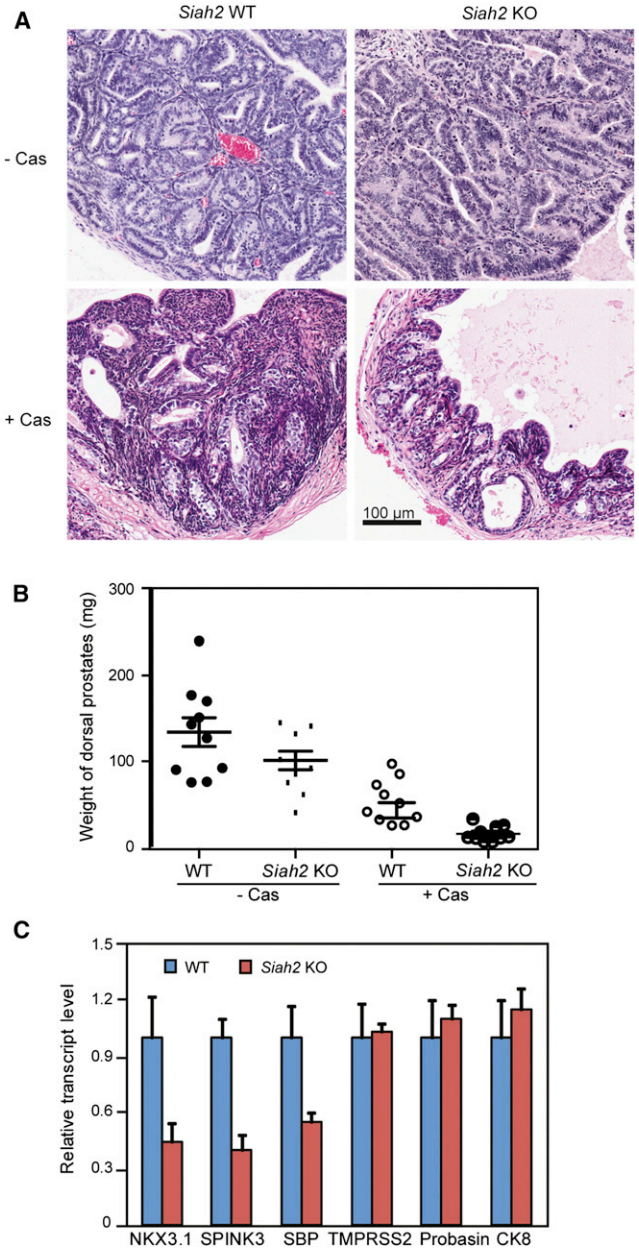

Figure 1. Siah2 Is Required for the Castration Sensitivity and Expression of Select AR Targets in the TRAMP Atypical Hyperplasia Model

(A) H&E staining of AH in the dorsal prostates of *Siah2*$^{+/+}$*;TRAMP* or *Siah2*$^{-/-}$*;TRAMP* mice before and after castration.

(B) The scatter plot showing the average weight of dorsal prostates microdissected from 5-month-old mice with or without 3-week castration (n = 10 for each group). p = 0.17 for WT versus Siah KO, − castration; p < 0.005 for WT versus Siah KO, + castration.

(C) Transcript levels of selected AR target genes in the prostates of *Siah2*$^{-/-}$*;TRAMP* mice after castration. RNA for qRT-PCR was isolated from the dorsal prostates of 5-month-old mice with 3 week castration. p < 0.01 for NKX3.1, p < 0.005 for SPINK3, p < 0.05 for SBP, p > 0.1 for TMPRSS2, probasin, or CK8. Data are mean ± SD.

See also Figure S1.

family of evolutionarily conserved RING finger E3 ubiquitin ligases. The Siah proteins regulate ubiquitination-dependent degradation of multiple substrates, including nuclear corepressor (NCOR1), β-catenin, TRAF2, α-ketoglutarate dehydrogenase, and Sprouty 2, and thus influence an array of regulatory functions such as the MAPK signaling, cell survival, and mitochondrial biogenesis (Kim et al., 2011; Nakayama et al., 2009). Siah1 and Siah2 also enhance the availability and activity of hypoxia-inducible factor (HIF-α) by mediating the ubiquitination and degradation of HIF-α-negative regulators, including PHD1/3, HIPK2, and FIH (Calzado et al., 2009; Fukuba et al., 2008; Nakayama et al., 2004). Here, we identify Siah2 as an E3 ligase that targets a select pool of chromatin-bound ARs, through which Siah2 controls the growth, survival, and tumorigenic capacity of PCa cells, especially under conditions of androgen deprivation.

RESULTS

Siah2 Deletion Increases the Castration Sensitivity of TRAMP Mice

We previously reported that crossing *Siah2*$^{-/-}$mice with *TRAMP* (transgenic adenocarcinoma of the mouse prostate) mice abolished the spontaneous formation of prostate NE tumors (Qi et al., 2010). In the TRAMP model, prostate-specific expression of SV40 T-antigen results in two types of lesions: NE carcinoma, found in the ventral lobe, and atypical hyperplasia (AH; often termed adenocarcinoma), which occurs in all lobes (Chiaverotti et al., 2008). To further investigate the possible role of Siah2 in the development of prostate tumors, we subjected *Siah2*$^{-/-}$*;TRAMP* mice to castration. As expected, castration caused shrinkage of AH in the dorsal prostate lobes of both genotypes (Figure 1A). However, the weight of dorsal prostate lobes was reduced approximately 10-fold in *Siah2*$^{-/-}$*;TRAMP* mice compared with 2.5-fold in the *Siah2*$^{+/+}$*;TRAMP* mice (Figure 1B). These results indicate that in *TRAMP* mice Siah2 deletion increased the sensitivity of AH to castration, implying that Siah2 may be required for AR signaling when androgen levels are low. Indeed, comparing expression of AR target genes in the dorsal prostate from *Siah2*$^{-/-}$*;TRAMP* mice with *Siah2*$^{+/+}$*;TRAMP* mice identified a greater reduction in transcript and protein levels of some AR target genes (i.e., *NKX3.1* and *SPINK3*, but not probasin and *TMPRSS2*; Figure 1C and Figure S1A available online), pointing to the possibility that Siah2 regulates the expression of a specific subset of AR target genes. The reduction in lesions observed in *Siah2*-deficient mice is not likely to be due to *probasin*-driven SV40 T-antigen expression, as the level of probasin transcripts and SV40 T-antigen protein were similar in *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$*;TRAMP* tissues (Figures 1C and S1B). In agreement, the dorsal prostates from both genotypes expressed similar transcript levels of the luminal marker CK8 (Figure 1C), suggesting that the reduced expression of AR target genes was not due to a change in cellular differentiation.

Siah2 Is Required for Expression of a Subset of AR Target Genes

To determine if Siah2 regulates AR activity in human PCa cells, we used shRNA to inhibit Siah2 expression in androgen-dependent LNCaP cells. Four of the 10 different Siah2 shRNAs

examined elicited 70%–90% knockdown (Figure S2A). To determine if Siah2 regulated transcription of PSA, a well-established AR target gene, LNCaP cells were cultured in medium supplemented with 5% charcoal stripped (CS)-fetal bovine serum (FBS) for 2 days and then stimulated with the synthetic androgen R1881 for 16 hr. Siah2 knockdown reduced PSA transcripts by 80%–90% in the absence of R1881 (Figure 2A) and by 50% in the presence of a physiologic level of R1881 (0.5 nM), consistent with changes reported upon knockdown of AR in LNCaP cells (Bao et al., 2008). In the presence of high levels of R1881 (10 nM), the degree of reduced PSA transcript was limited to 20% (Figure 2A). These results indicate that Siah2 is required for PSA transcription under conditions of both low and physiologic concentrations of androgen.

We next knocked down Siah2 in the androgen-independent PCa cell line, CWR22Rv1 (Rv1), which expresses constitutively active AR. Under androgen-deprivation conditions, Siah2 knockdown led to an 80% reduction in PSA transcript levels in Rv1 cells in the presence or absence of androgen (R1881; Figure 2B), confirming the importance of Siah2 for transcription of AR target genes.

Because Siah2 plays an important role in regulating the cellular response to hypoxia (Nakayama et al., 2004) and hypoxia has been reported to activate AR activity, we examined transcription of AR target genes under 1% O_2. Surprisingly, the relative change in PSA transcript levels upon Siah2 knockdown was similar under normoxia and hypoxia (Figures 2B and 2C). Notably, Siah2 knockdown reduced the transcript level of CA9, a HIF target gene, only under hypoxia (Figures 2B and 2C). These findings suggest that the effect of Siah2 on AR target gene expression is independent of its established role in the hypoxia response.

We next used qRT-PCR to compare the expression of representative androgen-responsive genes (ARGs) in LNCaP or Rv1 cells subjected to inhibition of AR or Siah2 expression. Siah2 knockdown in LNCaP or Rv1 cells reduced the expression of a subset of ARGs; transcripts of PSA, NKX3.1, PMEPA1, and SLC45A3 were reduced by Siah2 knockdown while TMPRSS2 and FKBP5 transcripts were unaffected (Figures 2D and 2E). In contrast, AR knockdown reduced the transcript levels of all these representative ARGs (Figures 2D, S2B, and S2C). These analyses confirmed that Siah2 modulates the expression of a specific pool of AR target genes in both LNCaP and Rv1 cells. To determine whether the effect of Siah2 on PSA transcripts is AR dependent, we knocked down Siah2 in LNCaP or Rv1 cells stably expressing AR shRNA. Although Siah2 knockdown reduced the PSA transcript level in control cells, it failed to do so in AR-knockdown cells (Figure 2F), suggesting that the Siah2 effect on PSA is AR dependent.

To evaluate the global effect of Siah2 on transcription of ARGs, we performed array-based gene expression analyses on Rv1 cells stably expressing Siah2 shRNA. Siah2 knockdown resulted in downregulation of 981 genes (>1.5-fold; Table S1). Gene network analysis, using the IPA software, was performed to identify possible enrichment of regulatory networks among the 981 genes. Notably, the AR-related genes were ranked among the top three transcriptional networks that were responsive to Siah2 inhibition (Figure 3A), with the other two being hypoxia- and HNF4A-related. Changes in hypoxia-response genes are consistent with the established role of Siah2 in control of HIF-1α expression, stability, and activity (Calzado et al., 2009; Fukuba et al., 2008; Nakayama et al., 2004). Further restricting the analysis to genes that are primarily associated with prostate cancer, we ranked the AR gene network as the primary one to be altered upon Siah2 knockdown (Figure 3B). These analyses point to AR as the major signaling pathway underlying Siah2-dependent gene expression in the Rv1 cells. About 10% (98/981) of Siah2-dependent genes were identified within the androgen-induced gene cluster in an ARG data set (Figure 3C; Table S1). Correspondingly, about 13% (98/759) of androgen-induced genes in the ARG data set were enriched within the Siah2-dependent expression cluster (Figure 3C). IPA analysis for molecular and cellular functions revealed that the 98 Siah2-dependent ARGs were associated with lipid metabolism, cell movement, and cell proliferation (Figures 3D and 3E), whereas the remaining 883 Siah2-dependent genes were associated with DNA replication/repair, and cell morphology and proliferation (Figure 3F). Independent analysis using the GO pathway software package confirmed lipid metabolism, steroid metabolism, and cholesterol metabolism as the primary pathways enriched in the 98 Siah2-dependent ARGs (Figure 3G; Table S2), whereas the remaining 883 Siah2-dependent genes were associated with oxidation, protein polymerization, and organic acid metabolism (Figure 3H; Table S2). Together, these gene expression analyses substantiate the role of Siah2 in the regulation of a subset of ARGs, while identifying distinct functional networks that are associated with the Siah2-dependent ARGs.

To determine whether the Siah2-regulated ARGs are important for the proliferation of PCa cells under the low androgen condition, we performed a siRNA screen in which we have targeted each of 98 Siah2-dependent ARGs. We identified 48 out of 98 siRNAs capable of inhibiting the proliferation of Rv1 cells grown in the CS-FBS medium by 10%–40% (Table S3). Notably, the most pronounced inhibition was observed by Siah2 siRNA. The siRNA screen also revealed that 8/16 Siah2-dependent ARGs involved in the metabolisms of lipids, cholesterol, and steroids (Figure 3E) were required for the proliferation and survival of Rv1 cells (Table S3). Since SREBF1, a master transcriptional regulator for lipid and cholesterol metabolism, was found among the Siah2-dependent ARGs, we further assessed the importance of SREBF1 for Siah2-dependent effects on Rv1 cells maintained in the CS-FBS medium. Significantly, inhibition of Rv1 cell proliferation upon Siah2 knockdown could be partially rescued upon re-expression of SREBF1 (Figures S3A and S3B). These findings substantiate the importance of the Siah2-AR regulatory axis in control of lipid metabolism for androgen-independent growth of Rv1 cells.

Comparison of the Siah2-dependent genes identified in our study with the published profiling arrays on prostate cancers revealed that 53 genes that were downregulated upon Siah2 knockdown in Rv1 cells were found to exhibit increased expression in the CRPC xenograft tumor model (Table S4) (Ettinger et al., 2004), and 44 of those genes were upregulated in high-grade prostate cancers (Table S5) (Taylor et al., 2010). Furthermore, analyses of profiling array data obtained from 35 CRPC and 58 primary PCa samples (Grasso et al., 2012) confirmed that 25% of Siah2-dependent ARGs identified in our current study were upregulated in CRPCs and enriched for genes

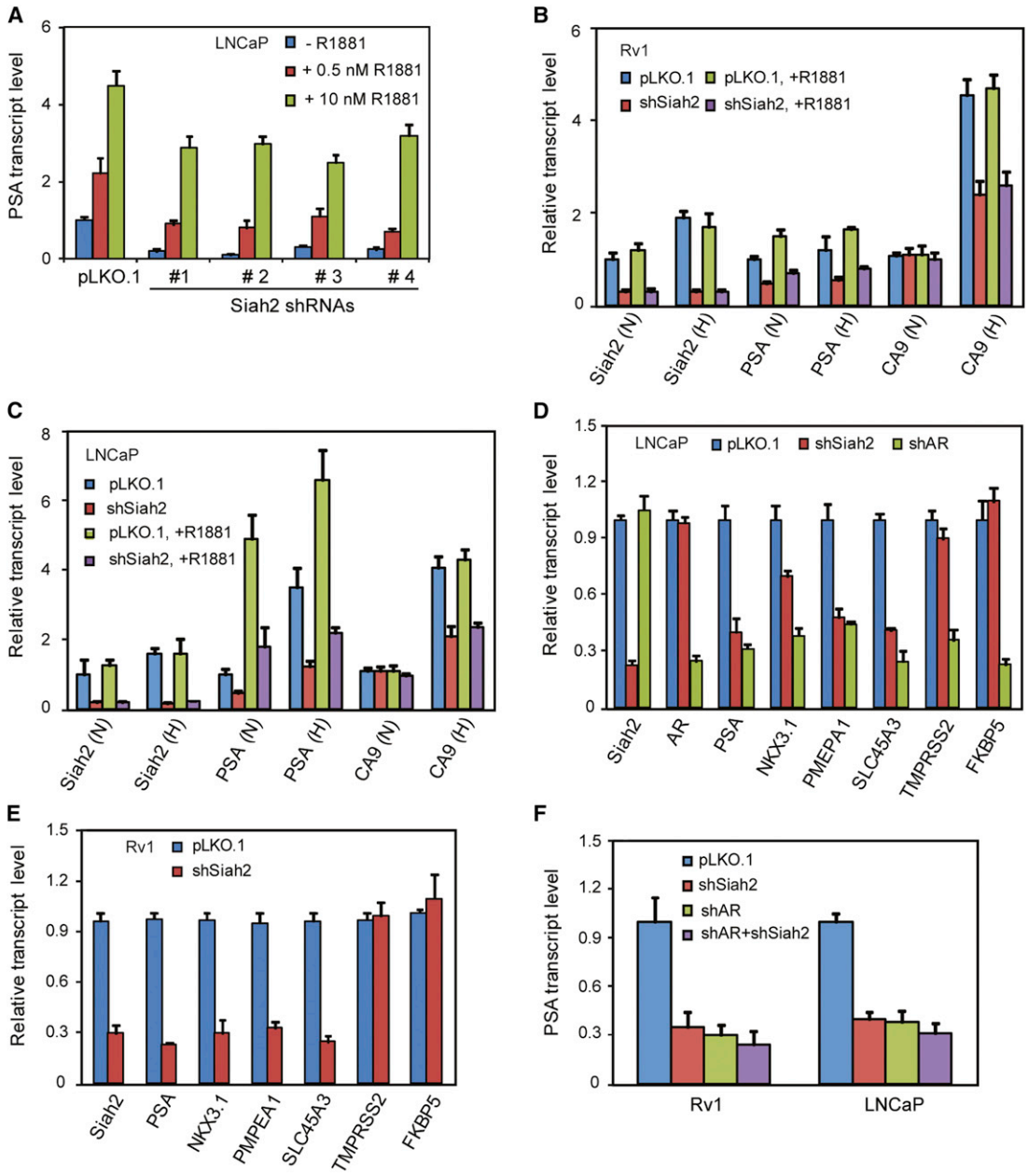

Figure 2. Siah2 Is Required for the Expression of Select AR Targets in Human Prostate Cancer Cells

(A) Effect of Siah2 knockdown in LNCaP cells on the PSA transcript levels. Siah2 was knocked down in LNCaP cells using four different Siah2 shRNAs. LNCaP transfectants were grown in medium containing 5% CS-FBS for 48 hr before treatment with 0.5 nM or 10 nM of synthetic androgen R1881 for 16 hr. The differences in PSA transcript levels between Siah2-knockdown and pLKO.1-transfected control cells were statistically significant ($p < 0.005$) in the presence and absence of androgen.

(B) Effect of Siah2 knockdown in Rv1 cells on the PSA transcript levels under normoxia or hypoxia. Rv1 cells transfected with Siah2 shRNA were grown in medium containing 5% CS-FBS for 48 hr and then treated for 16 hr with or without hypoxia or 10 nM R1881. Hypoxia did not increase the PSA transcript level in either pLKO.1 control or Siah2-knockdown cells ($p > 0.1$). N, normoxia; H, hypoxia.

(C) Effect of Siah2 knockdown in LNCaP cells on the PSA transcript levels under normoxia or hypoxia. The analysis was performed as for (B).

(D) qRT-PCR analysis of the indicated AR target genes in LNCaP cells transfected with shSiah2 or shAR vectors. Reduction in transcripts of PSA, NKX3.1, PMEPA1, and SLC45A3 by shSiah2 or shAR was statistically significant. Transcripts of TMPRSS2 or FKBP5 were reduced by shAR ($p < 0.01$) but not by shSiah2 ($p > 0.1$).

(E) qRT-PCR analysis of the indicated AR target genes in Rv1 cells transfected with shSiah2 vector. Reduction in transcripts of PSA, NKX3.1, PMEPA1, and SLC45A3 by shSiah2 was statistically significant ($p < 0.01$).

(F) qRT-PCR analysis of PSA transcripts in the indicated transfectants of LNCaP or Rv1 cells. The reduction of PSA transcript in the indicated knockdown cells was statistically significant ($p < 0.0005$). Data are mean ± SD.

See also Figure S2.

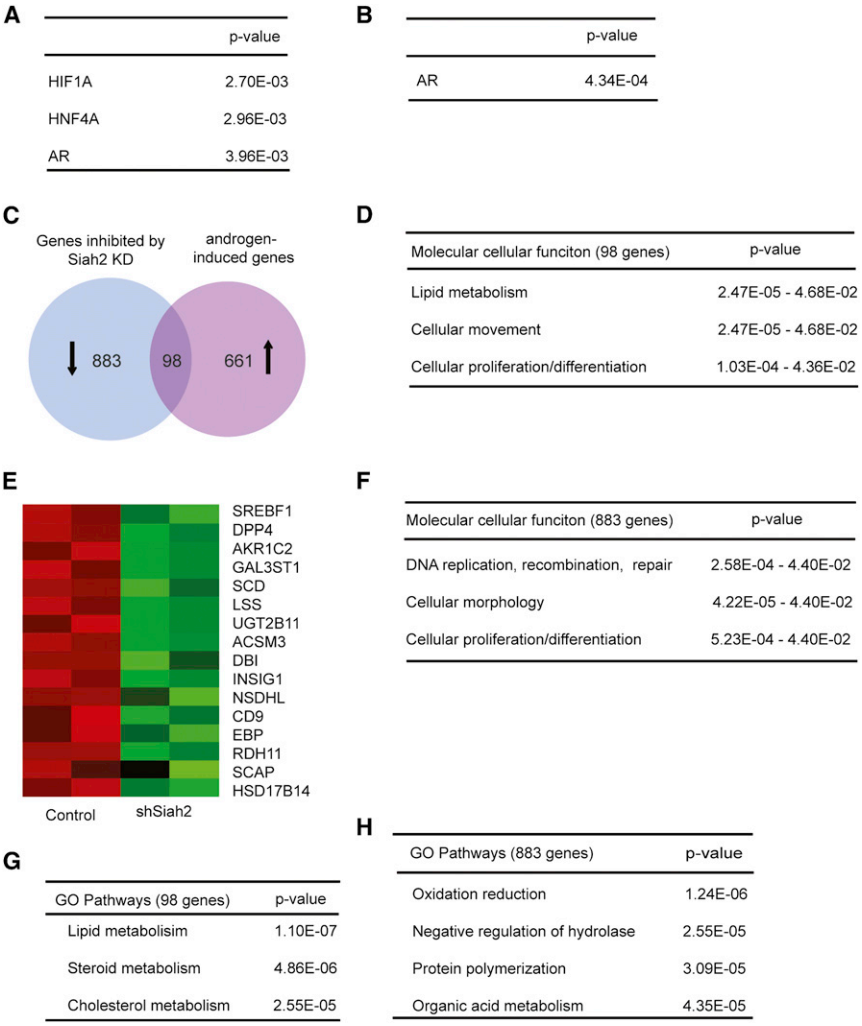

Figure 3. Bioinformatic Analyses of Siah2-Dependent Genes in Rv1 Cells

(A) Top three transcription factors predicted to underlie Siah2-dependent transcription by IPA. A total of 981 genes downregulated upon Siah2 knockdown were subjected to IPA analysis against canonical gene pathways.

(B) IPA analysis of transcription factors enriched within the 981 genes, as described in (A), against human prostate cancer cell data set.

(C) Venn diagram depicting overlap between genes downregulated by Siah2 knockdown and those induced by androgen signaling. The androgen-induced ARGs in prostate cancer cells were extracted from an ARG data set.

(D) The molecular and cellular functions of the 98 Siah2-dependent ARGs revealed by IPA analyses.

(E) Heatmap of the Siah2-dependet ARGs that regulate biosynthesis and metabolism of lipid, cholesterol, and steroids. Upregulated genes, red; downregulated genes, green.

(F) The molecular and cellular functions of the 883 genes (Siah2-dependent but AR-independent) revealed by IPA analyses.

(G) DAVID GOBP analyses of pathways enriched in the 98 Siah2-dependent ARGs.

(H) DAVID GOBP analyses of pathways enriched in the 833 genes (Siah2-dependent, but AR-independent).

See also Figure S3 and Tables S1, S2, S3, S4, S5, and S6.

involved in the biosynthesis of lipids, cholesterol, and steroids (Table S6). This observation substantiates the finding of metabolism and biosynthesis of lipids, cholesterol, and steroids as the primary ARGs regulated by Siah2 (Figures 3D and 3E). Importantly, Siah2 expression was found to be upregulated in the CRPC samples (Table S6), supporting a key role of Siah2 in CRPC. These results further confirm Siah2-dependent expression of a select subset of genes that are implicated in the progression and development of CRPC.

Siah2 Functions as an E3 Ubiquitin Ligase for AR

We next set to determine the mechanism underlying Siah2 effect on AR. First, we determined whether Siah2 and AR interacted by co-expressing AR with a wild-type (Siah2WT) or catalytically inactive RING mutant form of Siah2 (Siah2RM), which forms stable complexes with Siah2 substrates (Nakayama et al., 2004). Analysis of AR immunoprecipitates showed that Siah2RM or Siah2WT bound to AR protein (Figures 4A and S4A). Expression of Siah2WT, but not Siah2RM, reduced the levels of AR overexpressed in 293T cells (Figure 4B) or PC3 cells (Figure S4B). Overexpression of Siah2 in PCa cells also reduced the endogenous levels of AR (Figure S4C). Similarly, reduction of AR levels was observed following expression of WT but not the RING mutant form of Siah1a (S1aRM; Figure S4D). The effect of Siah2 on AR levels was partially blocked by treatment of 293T or PC3 cells with the proteasome inhibitor MG132 (Figures S4E and S4F), suggesting that Siah2 induces AR degradation via the ubiquitin-proteasome pathway. To determine if AR was polyubiquitinated by Siah2, we co-expressed Siah2, AR, and ubiquitin in 293T cells and found that Siah2WT, but not Siah2RM, induced AR polyubiquitination in the presence or absence of R1881 (Figure 4C). Similarly, purified GST-Siah2 induced polyubiquitination of AR in vitro (Figure S4G). Because polyubiquitination of AR can occur on lysine residues other than lysine 48 (K48) (Xu et al., 2009), we examined the topology of the AR ubiquitin chains by co-expressing AR, Siah2, and expression vectors for K48 or K63 mutant ubiquitin (Figure 4D). Siah2 expression was found to enhance polyubiquitination of AR in the presence of K63, but not K48, mutant ubiquitin, suggesting that Siah2 promotes K48-linked ubiquitination of AR. To determine whether Siah2 alters the half-life of AR, we performed cycloheximide chase assays in 293T cells ectopically expressing AR, with or without Siah2. Expression of Siah2 reduced the half-life of AR from 6 hr to less than 2 hr (Figure S4H). These findings suggest that Siah2 regulates AR stability by targeting it for ubiquitination-dependent degradation by the proteasomes.

We next mapped the AR domains required for Siah2 interaction by making domain-deletion mutants of AR and

co-expressing them with Siah2 in 293T cells. Western blot analyses indicated that AR mutants containing the AF2 domain were degraded by Siah2, whereas AF2-deficient mutants were resistant to Siah2-induced degradation (Figures S4I and S4J), suggesting that Siah2 interacts with the AF2 domain of AR. Indeed, GST-Siah2 was able to pull down the AF2, but not the AF1, domain of AR (Figure 4E). Siah2 consists of an N-terminal domain, a central RING domain/zinc finger domain, and a C-terminal substrate-binding domain (SBD) (Figure S4K). To map the Siah2 domain required for AR interaction, we generated truncation mutants of Siah2 and co-expressed them with AR in 293T cells. Immunoblotting revealed that both the Siah2 SBD and central RING domain/zinc finger domains interacted with AR (Figure 4F).

Because Siah2 interacts with the AR LBD, we tested whether the presence of androgen affects the AR–Siah2 interaction and whether Siah2 affects androgen–AR binding. The interaction between ectopic AR and Siah2 was unaffected by R1881 (Figure S4L), suggesting that ligand binding does not affect the AR–Siah2 interaction. Further, knockdown of Siah2 in Rv1 cells did not influence the affinity of [^{3}H]-labeled R1881 for the endogenous AR or its dissociation rate (Figure S4M). These observations indicate that ligand binding does not affect the AR–Siah2 interaction, nor does Siah2 affect the androgen–AR interaction.

AR Contains Two Major Siah2-Binding Sites

To identify the regions of the AR LBD that interacted with Siah2, we mutated AR with a series of single amino acid mutations that had been previously characterized in human PCa samples. The mutated ARs were individually co-expressed with Siah2RM in 293T cells, and the binding between AR mutants and Siah2RM was assessed by co-immunoprecipitation experiments.

AR constructs with the mutations R726L, Q798E, and F754L, all containing solvent-accessible side chains, showed the greatest reduction in Siah2 binding. R726L is clustered in the AF2 site with three other mutations (V715M, H874Y, and V730M) that cause moderate inhibition of Siah2 binding (Figures 4G–4I). Two additional mutations (L701H and T877A) in the AR ligand-binding pocket proximal to the AF2 site had moderate effects on Siah2 binding to AR.

The two remaining mutations that strongly reduced Siah2–AR binding (Q798E and F754L) are located in proximity in a hydrophobic cleft on the opposite side of the AR hinge region (Figure 4I). Interestingly, a mutation at V757, which lies just under this cleft, does not significantly decrease the Siah2–AR association, which points to its specific recognition of this cleft. In summary, the mutational analysis identified two solvent-accessible surfaces on AR LBD that are required for the interaction with Siah2.

To determine whether the Siah2–AR interaction is essential for AR transcriptional activity, we monitored the PSA promoter-driven luciferase activity in AR null PC3 cells transfected with AR mutants. Although knockdown of Siah2 reduced luciferase activity induced by WT AR or an AR mutant (G683A) capable of associating with Siah2 under both low and normal androgen conditions, it had limited effect on luciferase activity induced by AR mutants (R726L or Q798E) with markedly (70%) reduced Siah2-binding abilities (Figure 4J). Consistently, AR mutants R726L or Q798E showed impaired Siah2-dependent ubiquitination, compared with WT AR or G683A mutant AR (Figure S4N). These observations indicate that AR mutations that prevent Siah2-binding also prevent Siah2-mediated ubiquitination of AR and regulation of AR transcriptional activity, and further support the importance of the Siah2-AR association in the control of selected AR target genes, illustrated here by the PSA promoter.

Siah2 Is Required for Degradation of NCOR1-Bound AR on AREs of Selective AR Target Genes

To further understand Siah2 regulation of AR activity, we assessed whether knockdown of Siah2 affected levels of total, nuclear, or chromatin-bound AR in PCa cells. Significantly, Siah2 had no effect on any of these AR pools (Figures S5A–S5F). Overexpression of Siah2 in 293T cells did not affect the AR intermolecular N-terminal and C-terminal interaction (Figure S5G), which is known to regulate AR transcriptional activity. Because Siah2 knockdown reduced transcripts of specific AR target genes (Figure 2), we determined if Siah2 modulates AR binding to target gene promoters/enhancers. For this, we used *PSA*, *NKX3.1*, and *PMEPA1* as representative Siah2-regulated AR target genes and *TMPRSS2* as a Siah2-independent AR target gene. The results of ChIP assays confirmed androgen-dependent association of Siah2 with the AREs of *PSA*, *NKX3.1*, and *PMEPA1* in LNCaP cells, but not with ARE of *TMPRSS2* (Figure 5A). Binding of Siah2 to the AREs of *PSA*, *NKX3.1*, and *PMEPA1* was reduced by knockdown of AR or Siah2 (Figure 5A), suggesting that Siah2 was recruited to these chromatin regions by AR. Similar results were observed for the Siah2 ChIP assays performed in Rv1 cells (Figure 5B). Because NCOR1 is a known AR corepressor and a Siah2 substrate (Frasor et al., 2005; Zhang et al., 1998), we performed ChIP assays to determine whether Siah2 knockdown affected the binding of AR and NCOR1 to the AR target genes. Siah2 knockdown in LNCaP and Rv1 cells increased the level of AR and NCOR1 on the AREs of *PSA*, *NKX3.1*, and *PMEPA1* (Figures 5C, 5D, S5H, and S5I). As expected, binding of AR and NCOR1 to the ARE of *TMPRSS2* was unaffected by Siah2 knockdown (Figures 5D and S5J). Knockdown of Siah2 had no effect on the global level of AR (Figure S5A) or NCOR1 (Figure S5K). These results suggest that Siah2 is required for the degradation of AR and NCOR1, at selective AR target genes. To further evaluate this hypothesis, we performed additional ChIP assays with 29 randomly selected AR targets (16 Siah2-dependent and 13 Siah2-independent, based on our global gene expression analysis; Figure 3). ChIP was performed on Rv1 cells (pLKO.1 control versus Siah2 knockdown cells) using antibodies to Siah2, NCOR1, and AR, and the precipitated chromatins were subjected to qRT-PCR analyses for AREs of the 29 AR targets. Some of the AREs have been reported in the literature; for the remainder, we consulted published ChIP-seq or ChIP-on-chip studies and then validated AR binding to the AREs by ChIP-PCR analyses. These experiments revealed that Siah2 knockdown increased the levels of AR and NCOR1 bound to 11 of the 16 Siah2-dependent AR targets (68.75%) (Figures S5L and S5M), whereas none of the 13 Siah2-independent AR targets showed such change (data not shown). This analysis satisfied statistical power analyses, which indicated

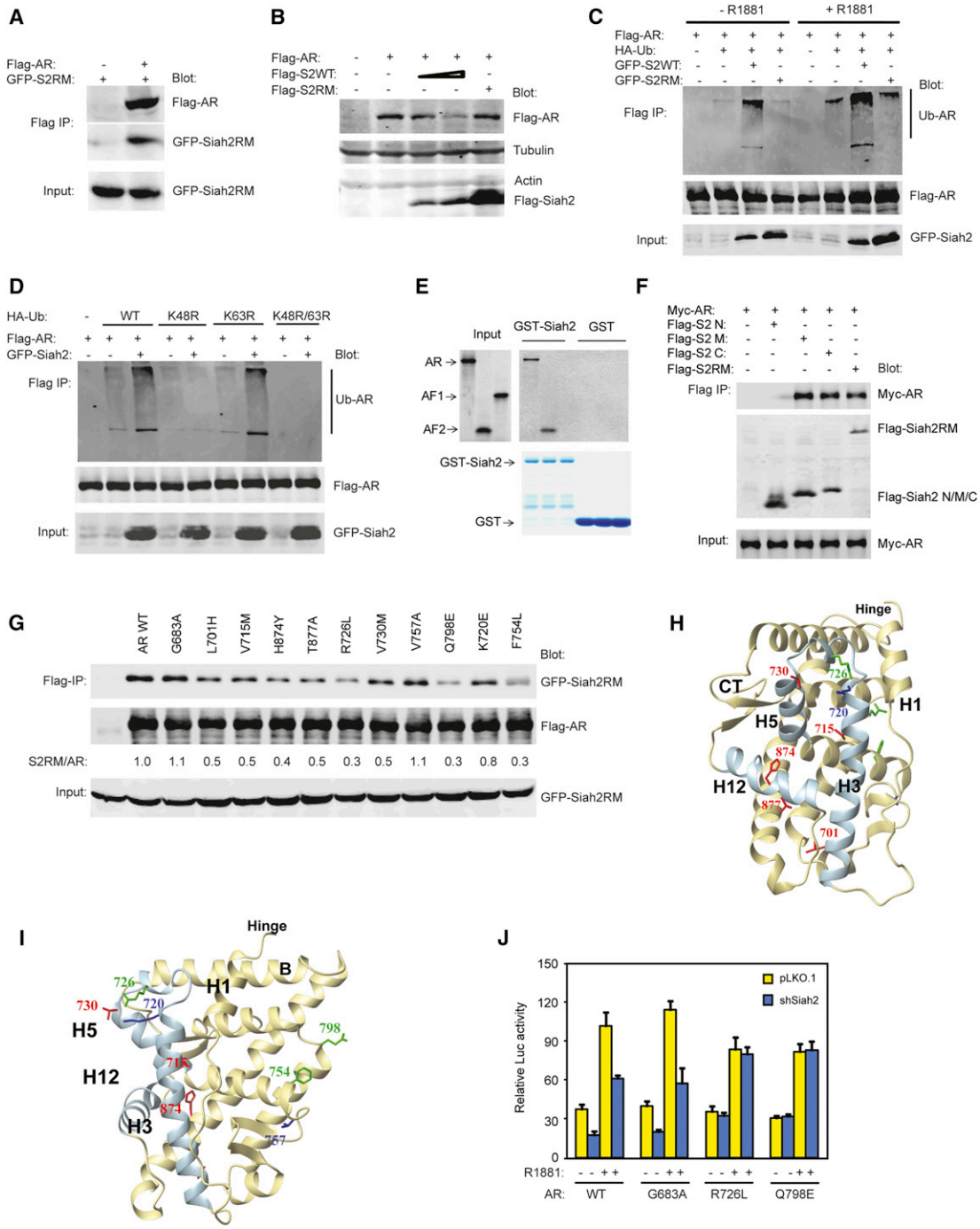

Figure 4. Siah2 Interacts with and Ubiquitinates AR for Proteasome-Dependent Degradation

(A) 293T cells were transfected with Flag-AR and GFP-Siah2RM for 24 hr before immunoprecipitation with anti-Flag M2 beads. Bound proteins were eluted and analyzed by western blotting with Flag or GFP antibodies.

(B) 293T cells were transfected with Flag-tagged AR and Siah2 (WT or RM) for 24 hr. Whole cell lysates were analyzed by western blotting with the antibodies of Flag, tubulin, and actin.

(C) 293T cells were transfected with Flag-AR, HA-Ub, and GFP-Siah2 (WT or RM) for 24 hr in the presence or absence of 10 nM R1881. Cells were treated with 20 μM MG132 for 5 hr and then Flag-AR was immunoprecipitated with M2 beads under denaturing conditions. The immunoprecipitates were analyzed by western blotting with HA or Flag antibody. The input of GFP-Siah2 was immunoblotted with GFP antibody.

(D) 293T cells were transfected with Flag-AR, HA-Ub (WT, K48 mutant, K63 mutant, and K48/K63 double mutant), and GFP-Siah2. The analysis was performed as described for (C).

(E) Flag-AF2 or-AF1 was in vitro translated (^{35}S labeling), purified, and incubated with GST-Siah2. Proteins bound to GST-Siah2 were analyzed by SDS-PAGE and phosphoimaging.

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a requirement for 12 genes per group to reach 95% confidence. Notably, Siah2 binding was demonstrated for nine of the 11 Siah2-dependent AR targets that exhibited increased AR/NCOR1 binding, compared with only one of the five Siah2-dependent AR targets that did not show increased AR/NCOR1 binding (Figure S5N). The association between Siah2 binding to AREs in control cells and the increased NCOR1/AR binding to these AREs upon Siah2 knockdown was statistically significant ($p < 0.05$, Fisher's exact test). Importantly, none of the Siah2-independent AR targets exhibited Siah2 binding (data not shown).

Collectively, the above data substantiate our conclusion that Siah2 targets the degradation of NCOR1-bound AR on a select group of AR target genes. Consistent with this, ChIP assays revealed that Siah2 knockdown reduced the level of ubiquitinated proteins on AREs of *PSA*, *NKX3.1*, and *PMEPA1*, but not *TMPRSS2* (Figure 5E). Notably, knockdown of NCOR1 attenuated the recruitment of Siah2 (Figures 5B and S5O) and the accumulation of AR on AREs of *PSA*, *NKX3.1*, and *PMEPA1* was further reduced upon Siah2 knockdown (Figures 5C, S5H, and S5I). Moreover, coprecipitation experiments showed a markedly higher association of Siah2 with the AR/NCOR1 complex than with AR alone (Figure 5F). These results suggest that the NCOR1-bound AR recruits Siah2 more efficiently. Indeed, knockdown of NCOR1 in Rv1 cells increased the transcripts of PSA, NKX3.1, and PMEPA1 in both control and Siah2-knockdown cells, but the effect was more pronounced in Siah2-knockdown cells (Figure 5G). Together, these results suggest that Siah2 may promote the AR transcriptional output via degradation of the transcriptionally-inactive AR (marked by NCOR1 binding) on the AREs of selective AR targets.

To investigate whether Siah2-mediated degradation of NCOR1-bound AR could affect binding of a co-activator to AR, we performed ChIP-reChIP assays that enable detection of multiple protein-protein interactions on specific chromatin regions. ChIP of Siah2 followed by reChIP of AR, NCOR1, and co-activator p300 revealed that Siah2 interacted with AR and NCOR1 but not with p300 on the PSA promoter (Figure 5H). Consistent with this, ChIP-reChIP assays revealed that NCOR1 and co-activator p300 are present in distinct AR complexes on the promoter of PSA gene (Figure 5I), and knockdown of NCOR1 increased the amount of p300 and acetylated histone H3 on the PSA promoter (Figures 5J and 5K), suggesting that competitive binding between p300 and NCOR1 to the AR modulates PSA promoter activity, consistent with findings from a previous report (Yoon and Wong, 2006). Interestingly, knockdown of Siah2 decreased the amount of p300 and acetylated histone H3 on the PSA promoter (Figures 5J and 5K), opposite to what was seen upon NCOR1 knockdown. Given that Siah2 knockdown increased the amount of NCOR1-bound AR on the PSA promoter (Figure 5C), these results suggest that Siah2-mediated degradation of NCOR1-bound AR (transcriptionally inactive) on PSA promoter allows the subsequent recruitment of p300-bound AR (transcriptionally active), leading to an increase in the transcription of PSA gene.

Knockdown of the E3 ubiquitin ligase Mdm2 in MEFs expressing ectopic AR has also been reported to disrupt AR turnover at the PSA promoter (Chymkowitch et al., 2011). To compare the effects of Siah2 and Mdm2 on AR transcriptional activity, we knocked down Mdm2 in Rv1 cells and examined transcripts of representative ARGs. The expression of some ARGs was regulated by both Siah2 and Mdm2 (e.g., *PSA*, *SLC45A3*), while others were regulated only by Siah2 (e.g., *NKX3.1*, *PMEPA1*) or Mdm2 (e.g., *TMPRSS2*), and some were unaffected by either protein (e.g., *FKBP5*) (Figures 2E and S5P). Knockdown of Mdm2 had little effect on the total level of AR (Figure S5Q), as had been observed with Siah2 knockdown. Additional regulatory layers (i.e., posttranslational modifications) are expected to exist in the control of AR by distinct ubiquitin ligases.

Modulation of AR Transcriptional Activity by Siah2 Is Required for Growth and Motility of Prostate Cancer Cells

We next evaluated the physiologic significance of Siah2 regulation of AR activity. Knockdown of Siah2 or AR significantly reduced proliferation of Rv1 and LNCaP cells (Figures 6A and 6B). However, knockdown of Siah2 in AR-knockdown cells did not further reduce cell proliferation (Figure 6B), suggesting that the Siah2 effect on cell proliferation may be AR dependent. Similarly, knockdown of Siah2 or AR in Rv1 or C4-2 cells (a castration-resistant subline of LNCaP) abolished anchorage-independent growth, as demonstrated by an inability to form colonies in soft agar (Figures 6C and 6D). In contrast, knockdown of Siah2 in AR-negative PC3 or DU145 cells (Figure S6A) had no apparent effect on cell growth (Figure 6A) or anchorage-independent growth (Figure S6B). Knockdown of Siah2 or AR in Rv1 cells reduced sphere formation in three-dimensional Matrigel to the levels seen with control Rv1 cells cultured under androgen-deprivation conditions (Figures 6E and S6C). Further, Siah2 or AR knockdown inhibited Rv1 and LNCaP cell motility in transwell

(F) Identification of the AR-interacting domains of Siah2. Myc-AR was cotransfected with Flag-tagged Siah2 fragments (N, N-terminal part; C, C-terminal part; M, middle part) in 293T cells. Siah2 fragments were immunoprecipitated with M2 beads and coprecipitated Myc-AR was analyzed by western blotting using Flag or AR antibody.

(G) 293T cells were cotransfected with GFP-Siah2RM and Flag-AR mutants as indicated. After 24 hr, immunoprecipitation was performed using M2 beads and samples were analyzed by western blotting using GFP or Flag antibody. The intensity ratios between GFP-S2RM and Flag-ARs are shown. The input of GFP-Siah2RM is shown at the bottom panel.

(H and I) Structural location of AR LBD mutants in WT AR. Ribbon presentation of the LBD with the AF2 site helices highlighted in blue and labeled in addition to the key helix H1 (H). The side chains of residues that were mutated are drawn as sticks, labeled, and colored green (strong), red (moderate), or blue (no effect) depending on their effect on Siah2 interaction. A 90° rotation of the model is shown to highlight the location of V757, F754, and Q798 (I).

(J) PC3 cells (pLKO.1 or shSiah2) were cotransfected with a PSA promoter *Gaussia* luciferase construct, a control *Cypridina* luciferase construct, and the mutant ARs indicated. Cells were grown in medium containing 5% CS-FBS for 48 hr and treated with 1 nM of R1881 for 16 hr. *Gaussia* luciferase activity was normalized to the *Cypridina* luciferase activity (n = 3). Data are mean ± SD. Siah2 knockdown reduced PSA promoter activity in cells expressing WT AR or G683 mutant AR in the absence or presence of R1881 ($p < 0.05$).

See also Figure S4.

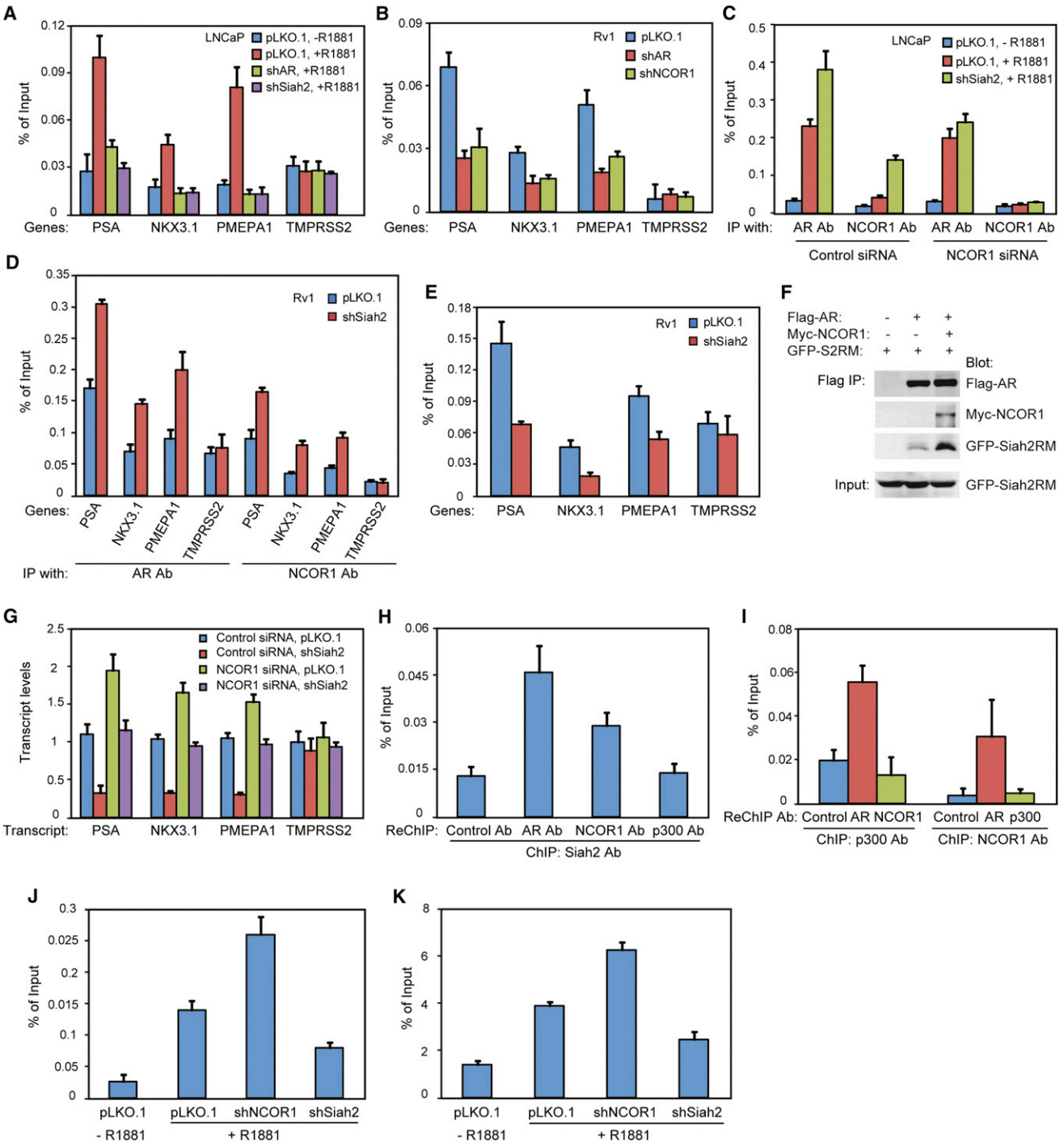

Figure 5. Siah2 Promotes the AR Transcriptional Activity by Targeting the NCOR1-Bound AR

(A) LNCaP cells stably transfected with control, AR shRNA, or Siah2 shRNA were grown in the absence or presence of 1 nM of R1881 for 12 hr, treated with 20 μM MG132 for 5 hr, and collected for ChIP assays using an anti-Siah2 antibody. Purified chromatin was analyzed by qPCR for the ARE regions of PSA, NKX3.1, PMEPA1, and TMPRSS2. Siah2 was enriched on AREs of PSA, NKX3.1, and PMEPA1 in the presence of R1881 ($p < 0.05$), but not on the ARE of TMPRSS2 gene ($p = 0.62$).

(B) Rv1 cells stably transfected with control, AR shRNA, or NCOR1 shRNA were grown in the normal growth medium, treated with 20 μM MG132 for 5 hr, and collected for the ChIP assays using anti-Siah2 antibody as described in (A). AR or NCOR1 knockdown affected Siah2 presence on the AREs of PSA, NKX3.1, and PMEPA1 ($p < 0.05$) and TMPRSS2 ($p > 0.1$).

(C) Effect of Siah2 knockdown in LNCaP cells on the association of AR and NCOR1 with the PSA promoter. ChIP assays were performed on LNCaP cells (pLKO.1 or shSiah2) using anti-AR or anti-NCOR1 antibodies and purified chromatins were analyzed by qPCR for the ARE region of PSA gene. Relative change in AR ($p < 0.05$) or NCOR1 ($p < 0.0005$) in the presence of R1881 or upon knockdown of Siah2, or NCOR1 ($p > 0.1$) is shown.

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assays, and this effect was amplified under androgen-deprivation conditions (Figures 6F, 6G, and S6D). In contrast, knockdown of Siah2 in AR-negative PC3 cells showed no effect on cell motility (Figure S6E). Collectively, these data strongly support a key role for Siah2 in the growth and motility of PCa cells, which is mediated at least partially through regulation of AR activity.

Siah2 Is Important for Growth of Castration-Resistant Prostate Tumors

Having established that Siah2 influences the growth and motility of AR-positive PCa cells in vitro, we next addressed its role in vivo. To establish CRPC, we used an orthotopic prostate tumor model in which the androgen-independent C4-2 cell line was injected into the dorsal prostate lobes of nude mice. We established C4-2 cells with stable knockdown of Siah2 and verified that they were similar to LNCaP Siah2-knockdown cells in both transcript levels of ARGs and in vitro growth characteristics (Figures S7A–S7C). Tumors derived from the Siah2-knockdown C4-2 cells were about half the size of those derived from control C4-2 cells (Figure 7A). Castration had no effect on tumor size of control cells, consistent with the fact that growth of C4-2 cells is androgen independent. Interestingly, the size of Siah2-knockdown tumors was reduced in response to castration (Figure 7A), suggesting that Siah2 contributed to the castration resistance of human PCa cells. qRT-PCR analyses revealed lower transcript levels of selective ARGs in the Siah2-knockdown C4-2 tumors, which were further lowered after castration (Figure 7B). In agreement, knockdown of AR reduced the C4-2 xenograft tumor size and PSA level in the castrated mice (Snoek et al., 2009). These findings suggest that Siah2-dependent regulation of AR activity may contribute to the castration resistance of C4-2 tumors.

Prostatic stromal cells from transgenic mice with a conditional knockout of the *tumor growth factor-β* (*TGF-β*) *receptor type II* in fibroblasts (*Tgfbr2*^{fspKO} and *Tgfbr2*^{ColTKO}) conferred resistance to castration in the prostate cancer models (Bhowmick et al., 2004). To further test the role of Siah2 in castration resistance, we knocked down Siah2 expression in a mouse prostate cancer cell line MPC3, knocked out for *Pten* and *Trp53*. Knockdown of Siah2 in MPC3 cells also reduced the transcription of selective AR targets (Figure S7D), characteristic of the Pten prostate cancer model (Mulholland et al., 2011). Tissue-recombination experiments combining WT or *Tgfbr2*-KO prostate stromal cells with MPC3 cells (control or Siah2 knockdown) were orthotopically grafted. The tumors grew diffusely in the mouse prostate and did not form a distinctive mass. The growth of MPC3 cells in vivo was evaluated by phosphorylated-histone H3, Ki67, and TUNEL staining. Knockdown of Siah2 in MPC3 cells recombined with WT prostate stromal cells led to reduced phospho-histone H3, Ki67, and TUNEL staining in castrated mice (Figures 7C, 7D, S7E, and S7F), indicative of Siah2′s role in the castration-resistant growth of MPC3 cells. Notably, co-culture of the Siah2-knockdown MPC3 cells with *Tgfbr2*-KO prostate stromal cells diminished the requirement of Siah2 for the proliferation of MPC3 cells in the castrated mice (Figures 7D and S7F). *Tgfbr2*-KO prostate stromal cells are known to promote the castration resistance of prostate epithelia via paracrine secretion of growth factors such as Wnt3a, interleukin-6 (IL-6), IL-1β, and human growth factor (HGF) (Bhowmick et al., 2004; Kiskowski et al., 2011), which can bypass the requirement for androgen signaling and thus Siah2 for the progression of MPC3 cells in the castrated mice. Together, these observations substantiate the role of Siah2 in the progression of CRPC.

Siah2 Expression Is Upregulated in Castration-Resistant Human Prostate Cancer

To verify the relevance of our findings to human PCa, we measured Siah2 expression in human castration-resistant prostate tumor samples. Siah2 was detected immunohistochemically in a PCa tissue microarray (TMA) containing representative samples of different Gleason stage tumors and castration-resistant tumors (Figures 7E–7G; Table S7). Siah2 showed a nuclear expression pattern in both the benign and the cancer cells. However, in the benign tissues, Siah2 was mostly present in basal cells, not luminal cells (Figure 7E). Compared to benign tissues, Siah2 expression was upregulated in PCa tissues, and

(D) Effect of Siah2 knockdown in Rv1 cells on the association of AR and NCOR1 with the AREs of PSA, NKX3.1 and PMEPA1. ChIP assays were performed on Rv1 cells (pLKO.1 control or shSiah2) using anti-AR or anti-NCOR1 antibodies and purified chromatins were analyzed by qPCR for ARE regions of PSA, NKX3.1, PMEPA1 and TMPRSS2. Level of AR ($p < 0.05$) and NCOR1 ($p < 0.05$) on the AREs of PSA, NKX3.1, and PMEPA1 upon Siah2 KD, compared with the ARE of TMPRSS2 ($p > 0.1$).
(E) Effect of Siah2 knockdown in Rv1 cells on the association of ubiquitinated proteins with the AREs of PSA, NKX3.1, and PMEPA1. ChIP assays were performed on Rv1 cells (pLKO.1 control or shSiah2) using anti-ubiquitin antibody and analyzed as described in (D). Level of ubiquitinated proteins upon Siah2 KD on the ARE regions of PSA, NKX3.1, and PMEPA1 ($p < 0.05$), compared with the ARE on TMPRSS2 gene ($p = 0.55$).
(F) 293T cells were transfected with Flag-AR or Flag-AR/myc-NCOR1, which were isolated by purification on M2 beads and then incubated with 293T lysates expressing GFP-Siah2RM. After three washes, the proteins were eluted and analyzed by western blotting using the antibodies of Flag, Myc or GFP.
(G) qRT-PCR of the indicated genes was performed on Rv1 cells (pLKO.1 or shSiah2) transfected with NCOR1 siRNA. Shown is the change in transcripts of PSA, NKX3.1, and PMEPA1 in pLKO.1 or Siah2 KD cells by the NCOR1 siRNA ($p < 0.05$).
(H) LNCaP cells were treated with 1 nM of R1881 for 12 hr, 20 μM MG132 for 5 hr, and collected for the first round of IP (ChIP) using Siah2 antibody. The eluates of the first IP were used for the second round of IP (reChIP) using antibodies indicated. The purified chromatins by the reChIP were analyzed by qPCR for the ARE region of PSA promoter. Relative enrichment by AR or NCOR1 antibody ($p < 0.05$), or p300 antibody ($p = 0.76$) is shown.
(I) LNCaP cells were treated with 1 nM of R1881 for 12 hr, and collected for the first round of IP (ChIP) using p300 (left columns) or NCOR1 (right columns) antibody followed by the second round of IP (reChIP) using the antibodies indicated, and analyzed by qPCR for the ARE region of PSA gene. Enrichment of reChIP by AR antibody is shown ($p < 0.05$).
(J) LNCaP cells subjected to NCOR1 or Siah2 KD were treated with 1 nM of R1881 for 12 hr, collected for the ChIP assays using p300 antibody, and analyzed by qPCR of the ARE region of PSA promoter. p300 enrichment upon NCOR1 or Siah2 KD ($p < 0.05$) is shown.
(K) ChIP assays using acetylated histone H3 antibody were performed on LNCaP cells as described in J. Shown is the relative enrichment of acetylated histone H3 antibody on PSA promoter upon NCOR1 or Siah2 knockdown ($p < 0.05$). Data are mean ± SD.
See also Figure S5.

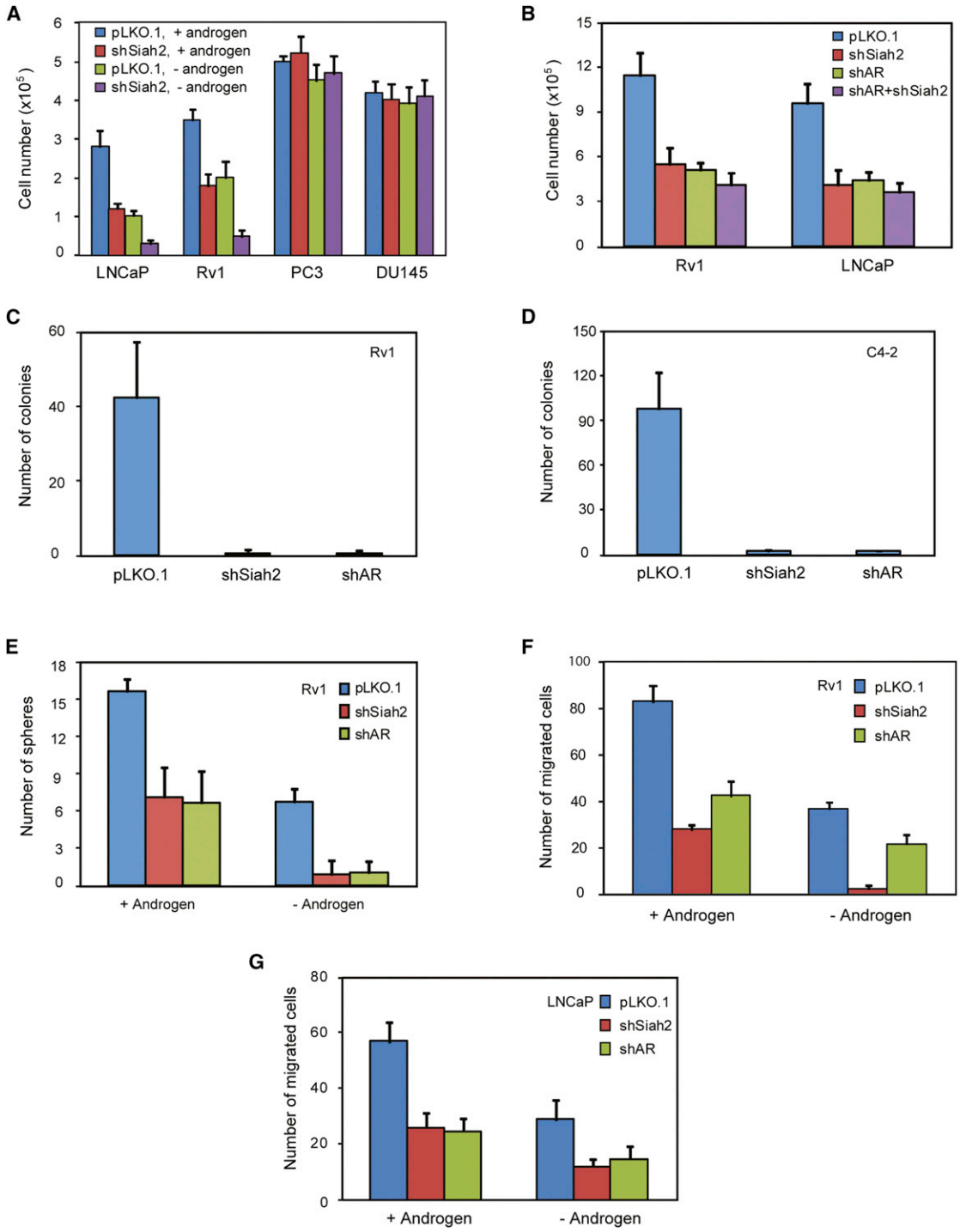

Figure 6. The Siah2 Effect on AR for the Proliferation and Motility of PCa Cells

(A) Effect of Siah2 knockdown on the growth of human PCa cells. LNCaP, Rv1, PC3, or DU145 cells were stably transfected with Siah2 shRNA or control pLKO.1 vector. Equal numbers of cells (1×10^4) were seeded in 12-well plates in medium containing 5% FBS (+androgen) or 5% CS-FBS (−androgen) and cells were counted after 5 days (n = 3). Change in cell growth upon Siah2 KD in LNCaP or Rv1 cells was maintained in the presence or absence of androgen ($p < 0.05$).

(B) Siah2 or AR was stably knocked down individually or in combination in LNCaP or Rv1 cells. Equal numbers of cells (5×10^4) were grown in six-well plates and cells were counted after 5 days (n = 3). Change in growth of Rv1 ($p < 0.01$) or LNCaP ($p < 0.005$) is shown.

(C) Knockdown of Siah2 or AR in Rv1 cells on the colony formation in soft agar assays. Cells (pKLO.1 control, shSiah2, or shAR) were maintained in soft agar for 3 weeks before staining with p-iodonitrotetrazolium violet. The number of colonies per field was quantified ($p < 0.0001$).

(D) Siah2 or AR KD effect on colony formation of C4-2 cells ($p < 0.00005$), was carried out as detailed in (C).

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interestingly, Siah2 upregulation was seen across Gleason stages within this cohort (Figure 7F). Significantly, levels of Siah2 expression were closely associated with ADT and development of CRPC, reflected in reduction of Siah2 levels after ADT and elevation of Siah2 levels in CRPC (Figure 7G). Interestingly the level of Siah2 protein in human CRPC resembles the changes seen in Siah2 transcripts in the human CRPC xenograft model; while the level of Siah2 transcripts was reduced upon castration, it increased during development of CRPC (Figure S7G). Notably, whereas Siah2 level is associated with clinical recurrence upon ADT, it is not associated with recurrence after prostatectomy (Figure 7H). Consistent with the upregulation of Siah2 in CRPCs, the endogenous Siah2 level is higher in the two androgen-independent PCa cell lines C4-2 and Rv1 than in the androgen-dependent LNCaP cells or the AR null PC3 cells (Figure S7H). Immunohistochemical staining of CLGN and ACPP (Siah2-dependent AR targets) in the human PCa tumor microarray revealed their elevated expression (~30% increase, $p < 0.05$) in CRPCs compared with the naive PCa samples (Figure S7I, data not shown). The latter substantiate the significance of Siah2-dependent AR targets in CRPCs.

DISCUSSION

Understanding the mechanisms underlying AR activity is important for the development of effective therapeutic modalities to treat CRPC. Here, we identified an undisclosed facet of AR regulation and demonstrated its consequences for the growth of castration-resistant prostate tumors. Our studies reveal that the E3 ubiquitin ligase Siah2 regulates a subset of AR, bound to the corepressor NCOR1, resulting in removal of the transcriptionally-inactive AR from chromatin. The specificity of Siah2 for the NCOR1-bound AR is achieved through Siah2 interaction with two accessible surfaces on AR, concomitant with its interaction with the AR/NCOR1 complex. Siah2-dependent removal of NCOR1-bound AR allows the binding of p300-bound AR to the AREs of PSA gene. Collectively, our findings suggest a model whereby Siah2 recognition and degradation of a specific inactive AR pool enables recycling of AR, whereby inactive AR-NCOR1 is replaced with active AR-p300, thereby sustaining constitutive AR-dependent transcription from selected promoters/enhancers.

The finding that Siah2 ubiquitin ligase controls a subset of AR is consistent with the concept that some transcription factors are removed from target promoters via the ubiquitin-proteasome pathway to allow binding of new factors and continued transcription (Muratani and Tansey, 2003). A similar mechanism has been proposed for regulation of PSA by Mdm2 (Chymkowitch et al., 2011), and interestingly, both Mdm2 and Siah2 reportedly regulate HIPK2 stability (Calzado et al., 2009; Rinaldo et al., 2007). Thus, the interplay between these two ligases may dictate the duration, level, and/or specificity of transcriptional output. It is equally plausible that Siah2 and Mdm2 play distinct roles in AR turnover for different pools of AR target genes, or under varying physiologic conditions. We favor the latter possibility, which is supported by the observation that Siah2 and Mdm2 regulate different AR targets (Figures 2E and S5P). Although we provide evidence that Siah2 contributes to AR turnover on the AREs of selective AR targets, we cannot exclude the possibility that it regulates AR activity through additional mechanisms, such as promoting recruitment of co-activators or degrading/displacing corepressors.

How do the Siah2-regulated ARGs contribute to CRPC? Our gene expression profiling studies point to Siah2-dependent regulation of AR target genes associated with sterol and lipid metabolism. These findings were confirmed by qPCR analyses in PCa cells and identification of this gene cluster in independent data sets reported for PCa and CRPC. Of those, a master regulator of lipid metabolism, SREBF1, a Siah2-dependent ARG, plays an important role in the proliferation of Rv1 cells under low androgen condition (Figure S3B) and is upregulated in CRPC (Table S6). SREBF1 induces the expression of enzymes involved in fatty acid and cholesterol synthesis, thereby providing CRPC cells with fatty acids for energy and membrane synthesis, and cholesterol for intratumoral de novo synthesis of androgens (Ettinger et al., 2004; Locke et al., 2008). Among the enzymes underlying the intratumoral androgen metabolism are AKR1C2, AKR1C3, and UGT2B15, which are upregulated in CRPC (Cai et al., 2011; Stanbrough et al., 2006), consistent with their downregulation in the Siah2-knockdown Rv1 cells.

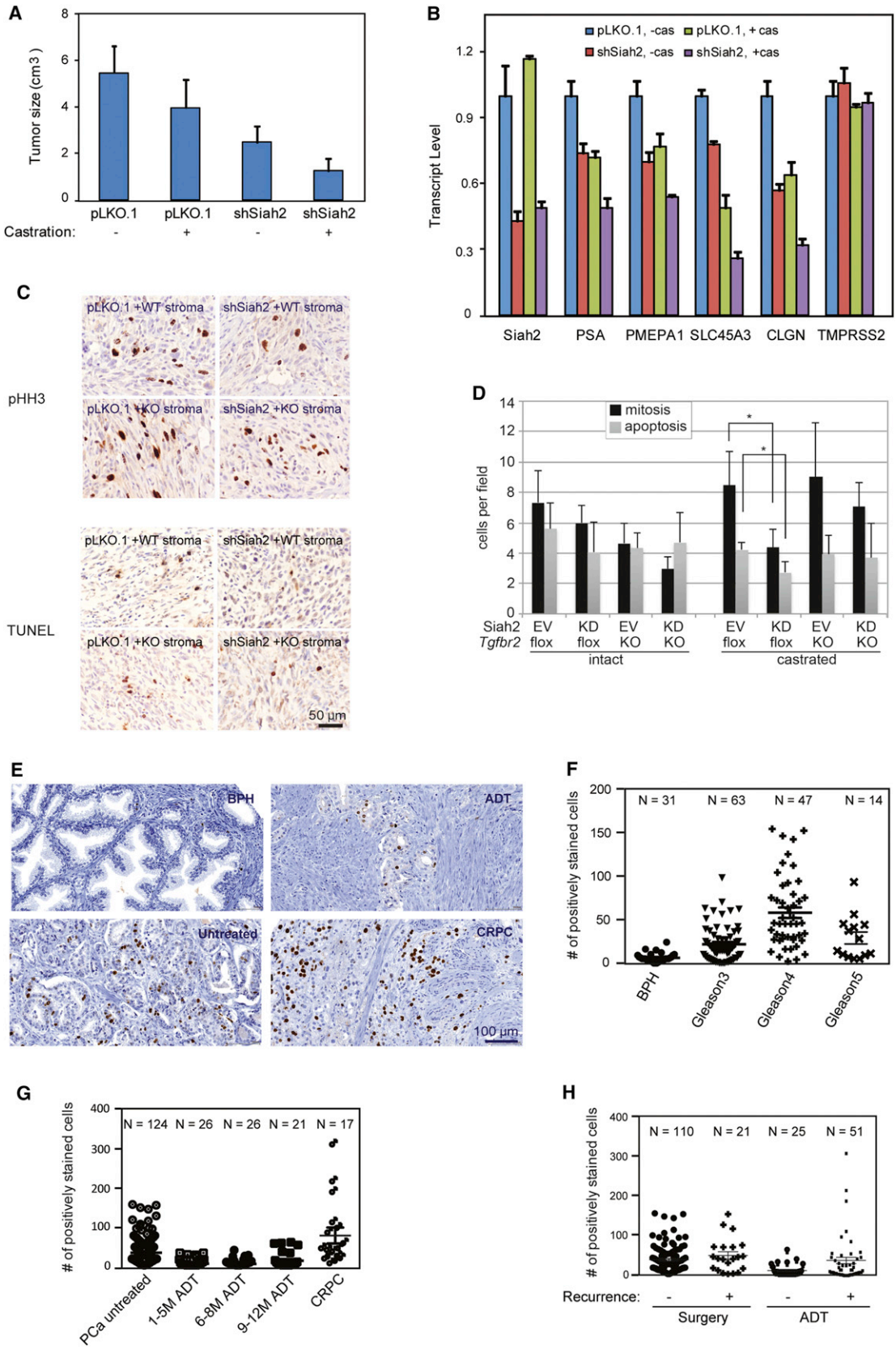
We previously reported that Siah2-dependent regulation of HIF was important to the neuroendocrine differentiation (NED) of PCa cells under chronic hypoxic conditions (Qi et al., 2010). Here, we show that Siah2-dependent regulation of AR activity is equivalent under normoxia and hypoxia. Notably, NED foci of prostate cancer express little or no AR protein (Huang et al., 2006), suggesting that the effect of Siah2 on AR activity is not applicable for NED foci, where the Siah2 contribution is mediated through its concerted regulation of HIF-1α and FoxA2 (Qi et al., 2010). Siah2 thus elicit distinct effects in different PCa cell populations: it enhances HIF activity in NED foci and promotes AR activity in PCa cells surrounding these foci. Importantly, through independent mechanisms in each of these cell populations, Siah2 contributes to the castration resistance of PCa. Thus, the role played by Siah2 in controlling AR signaling, castration resistance, and NED of PCa makes it a promising target for PCa therapy, either alone or in combination with other therapeutic approaches.

EXPERIMENTAL PROCEDURES

Prostate Tumor Samples

A total of 194 prostate cancer specimens were obtained from the Vancouver Prostate Tissue Bank under approval by Clinical Research Ethics Board

(E and F) Rv1 cells (pLKO.1, shSiah2, and shAR) were grown in the three-dimensional Matrigel in the presence (5% FBS) or absence (5% CS-FBS) of androgen for 1 week (E). The number of spheres (>25 μm in diameter) grown in the presence ($p < 0.05$) or absence ($p < 0.005$) of androgen is shown (F). Rv1 cells (pLKO.1, shSiah2, and shAR) were subjected to transwell assays in the presence (5% FBS; $p < 0.0005$) or absence (5% CS-FBS; $p < 0.05$) of androgen for 24 hr.
(G) Migration of LNCaP cells upon KD of Siah2 or AR in the presence ($p < 0.0005$) or absence ($p < 0.001$) of androgen, monitored using the transwell assays as described in (F). Data are mean ± SD.
See also Figure S6.



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(# H09-01628; informed consent was obtained from all subjects). All the specimens were from radical prostatectomy except the 12 CRPC samples, which were obtained from transurethral resections of prostate. The hematoxylin and eosin (H&E) slides were reviewed and the desired areas were marked. Three TMAs were manually constructed (Beecher Instruments, MD, USA) by punching duplicate cores of 1 mm from each sample.

Animal Studies

Animals were housed in the Sanford-Burnham Medical Research Institute (SBMRI) animal facility, or Cedars-Sinai Medical Center (CSMC). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC # 55545 and 56155 at SBMRI and IACUC # 3679 at CSMC) and were conducted following the Institute's animal policy in accordance with National Institutes of Health (NIH) guidelines.

Cell Lines

LNCaP, C4-2, PC3, DU145, and CWR22 Rv1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

Generation of Siah2$^{-/-}$;TRAMP Mice

Siah2$^{+/-}$ mice (129/SvJ background) were crossed with *TRAMP* transgenic mice (C57BL/6 background) to obtain *Siah2*$^{+/-}$*;TRAMP* mice. Female *Siah2*$^{+/-}$*;TRAMP* mice were crossed with male *Siah2*$^{+/-}$ mice to generate mice of the genotypes *Siah2*$^{+/+}$*;TRAMP*, *Siah2*$^{+/-}$*;TRAMP*, and *Siah2*$^{-/-}$*;TRAMP* (Qi et al., 2010).

Antibodies and Reagents

Antibodies to AR, p300, ubiquitin, GFP, myc, Mdm2, tubulin, actin (Santa Cruz), NCOR1, Histone H3, GFP, NKX3.1, Ki67, SV40 T-antigen (Abcam), acetylated histone H3 (anti-AcH3-K9, Upstate Biotechnology), Siah2 (Novus Biologicals), ACPP (Pierce), CLGN (Abgent), phospho-histone H3, SPINK3 (cell signaling), and Flag (Sigma) were used according to the manufacturers' recommendations.

Statistical Analysis

The data were analyzed by Student's t test, one-way ANOVA, or Fisher's exact test. A p value < 0.05 was considered statistically significant.

ACCESSION NUMBER

The GEO accession number for the microarray data reported in this paper is GSE38851.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, seven tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.02.016.

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Figure 7. Siah2 Promotes the Growth of CRPC Orthotopic Tumors, and Its Expression Is Elevated in Human CRPC Samples

(A) Orthotopic tumor size of C4-2 cells expressing Siah2 shRNA with or without castration. C4-2 cells (pLKO.1 or shSiah2) were injected into the dorsal prostates of nude mice and 3 weeks later one group of mice was castrated for 4 weeks. The size of tumors formed was quantified (n = 6 for each group). $p < 0.005$ for pLKO.1, − castration versus shSiah2, − castration; $p < 0.01$ for shSiah2, − castration versus shSiah2, + castration; $p = 0.16$ for pLKO.1, − castration versus pLKO.1, + castration.

(B) qRT-PCR analysis of the indicated genes on RNA samples extracted from the orthotopic tumors. Change in transcripts level for PSA, PMEPA1, SLC45A3, and CLGN upon Siah2 KD with or without castration ($p < 0.05$).

(C) The phospho-histone and TUNEL staining on the MPC3 tumor tissues collected from the indicated mice after castration. MPC3 pLKO.1 cells or Siah2-KD cells were recombined with WT or *Tgfr2* KO prostate stromal cells for an orthotopic injection. One month after injection, mice were castrated, and 4 days later tissues were collected and subjected to IHC analyses.

(D) Quantification of phospho-histone and TUNEL staining shown in (C).

(E) Representative images of Siah2 IHC staining on the PCa TMA. BPH, benign prostate hyperplasia.

(F) Quantification of Siah2 IHC staining on the BPH and PCa samples of different Gleason scores. ANOVA with Tukey's multiple comparison indicates $p < 0.0001$, except Gleason3 versus Gleason5 (lower number of cases in Gleason5 group).

(G) Quantification of Siah2 IHC staining on the PCa samples without or with ADT or the CRPC samples. ANOVA with posthoc results indicates $p < 0.0001$ for CRPC compared with all other groups and for untreated PCa compared with all other groups.

(H) Quantification of Siah2 staining on the PCa samples after prostatectomy or ADT (with or without clinical recurrence). ANOVA analyses of Siah2 staining between recurrent and nonrecurrent cases in samples after ADT ($p < 0.0001$), and between recurrent and nonrecurrent cases in samples obtained after prostatectomy ($p < 0.1$). Data are mean ± SD.

See also Figure S7 and Table S7.

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