The steroidogenic enzyme AKR1C3 regulates stability of the ubiquitin ligase Siah2 in prostate cancer cells

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Running Title: Regulation of Siah2 by AKR1C3 in prostate cancer

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Capsule

**Background:** Ubiquitin ligase Siah2 promotes activity of androgen receptor (AR) in prostate cancer cells.

**Results:** The steroidogenic enzyme AKR1C3 binds and stabilizes Siah2 by blocking Siah2 self-ubiquitination and degradation.

**Conclusion:** We identified a catalytic-independent role for AKR1C3 on AR activity via Siah2.

**Significance:** The findings may provide new targets for development of AKR1C3 inhibitors as prostate cancer therapy.

Abstract

Re-activation of androgen receptor (AR) activity is the main driver for development of castration-resistant prostate cancer (CRPC). We previously reported that the ubiquitin ligase Siah2 enhanced AR transcriptional activity and prostate cancer cell growth. Among the genes we found to be regulated by Siah2 was AKR1C3, which is a key androgen biosynthetic enzyme implicated in CRPC development. Here, we found that Siah2 inhibition in CWR22Rv1 prostate cancer cells decreased AKR1C3 expression as well as intracellular androgen levels, concomitant with inhibition of cell growth in vitro and in orthotopic prostate tumors. Re-expression of either wild-type or catalytically-inactive forms of AKR1C3 partially rescued AR activity and growth defects in Siah2 knockdown cells, suggesting a nonenzymatic role for AKR1C3 in these outcomes. Unexpectedly, AKR1C3 re-expression in Siah2 knockdown cells elevated Siah2 protein levels, whereas AKR1C3 knockdown had the opposite effect. We further found that AKR1C3 can bind Siah2 and inhibit its self-ubiquitination and degradation, thereby increasing Siah2 protein levels. We observed parallel expression of Siah2 and AKR1C3 in human prostate cancer tissues. Collectively, our findings identify a new role for AKR1C3 in regulating Siah2 stability and thus enhancing Siah2-dependent regulation of AR activity in prostate cancer cells.

Introduction

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer-related mortality among men in developed countries (1). Since androgens stimulate prostate cancer growth, standard treatment for metastatic PCa is androgen deprivation therapy (ADT), which is designed to block androgen receptor (AR) activity. AR belongs to the nuclear receptor superfamily, and primary AR ligands include dihydrotestosterone (DHT) and testosterone (T). Upon androgen binding, AR translocates to the nucleus and regulates gene expression, promoting PCa development and progression. Although advanced PCa initially responds well to ADT, the disease invariably relapses and progresses to a stage known as castration-resistant prostate cancer (CRPC). Understanding mechanisms that contribute to CRPC development and developing effective treatments for this disease state remain major challenges for basic and clinical research.

Re-activation of AR signaling is believed to drive the development of CRPC (2). AR becomes active in CRPC via changes in expression or activity of either AR itself or its ligands. Mechanisms underlying the former include overexpression, mutation, or formation of splicing variants of AR (3-5), while ligand availability is regulated via intratumoral androgen biosynthesis (6). Normally, testosterone (T) is produced in the testis and converted to the more potent dihydrotestosterone (DHT) in the prostate. ADT inhibits T production and release from the testis, significantly lowering serum T levels. However, T and DHT levels do not decrease significantly in CRPC cells, suggesting that these cells can synthesize androgens (7). Inhibitors (e.g., abiraterone) targeting the androgen biosynthetic enzyme CYP17 have shown significant activity in patients with CRPC (8,9).

The Siah family proteins are RING finger E3 ubiquitin ligases comprised of Siah1 and Siah2 in humans. Siah proteins induce ubiquitination and subsequent degradation of several substrates, and thus regulate numerous signaling pathways and biological processes (10). Like other ubiquitin ligases (11), Siah can also self-ubiquitinate and promote its own degradation through the ubiquitin-proteasome
pathway (12,13). Thus, Siah proteins are generally present at very low levels in cells. Siah2 reportedly plays a tumor-promoting role, and unregulated Siah2 activity can promote development and progression of lung, pancreatic, skin, breast, and prostate cancers (14-18). Our recent study revealed an important role for Siah2 in regulating AR activity and implicated it in CRPC development. In this context, Siah2 induces degradation of transcriptionally inactive AR bound to the corepressor NCOR1 (AR-NCOR1 complex) on specific AR target genes, allowing subsequent recruitment of transcriptionally active (coactivator-bound) AR to drive target gene transcription (19). Bioinformatic analyses of profiling array data suggest that androgen biosynthesis is a top function for Siah2-dependent genes, which include those encoding enzymes catalyzing androgen biosynthesis and metabolic activities, such as aldo-keto reductase 1C3 (AKR1C3), HSD17B8, HSD17B14, AKR1C2 and UGT2B15 (19). Of note, Siah2-dependent transcripts encoding such enzymes are reportedly upregulated in human CRPC samples (20,21).

AKR1C3 catalyzes reduction of two substrates: the weak androgen androstanedione to generate T and 5α-androstanedione to produce DHT (22,23). AKR1C3 is highly upregulated at mRNA and protein levels in high-grade PCa, recurrent PCa and CRPC tumor samples (20,21,24-26). A recent study revealed that AKR1C3 contributes to resistance of PCa cells to the AR antagonist enzalutamide (also known as MDV3100) by enhancing intratumoral androgen biosynthesis (27). Several selective inhibitors targeting AKR1C3 catalytic activity have been developed (28-31), although their effect on CRPC remains to be determined.

Given its role in intratumoral androgen biosynthesis, we asked whether AKR1C3 enzymatic function is required for Siah2-dependent regulation of AR activity and PCa growth. Using CWR22Rv1 cells (hereafter referred to as Rv1 cells) as a model, we found that AKR1C3 plays a positive regulatory role in Siah2-dependent AR signaling and growth of prostate cancer cells. Interestingly, we identified a catalytically-independent function of AKR1C3 in Siah2-dependent AR activity whereby AKR1C3 increases Siah2 stability by inhibiting Siah2 self-ubiquitination and degradation. Our findings suggest that non-catalytic AKR1C3 activity should be considered in developing AKR1C3 inhibitors as potential therapy for prostate cancer.

**Experimental Procedures**

**Antibodies and reagents.** The following antibodies were used according to manufacturers’ recommendations: AR, ubiquitin, HA, GFP, GST, His, Myc, and tubulin (Santa Cruz); Siah2 and NCOR1 (Abcam); AR (EMD Millipore); and Siah2, AKR1C3, Flag and actin (Sigma).

**Cell lines.** LNCaP, PC3 and DU145 cells were purchased from American Type Culture Collection (ATCC). Rv1 cells were kindly provided by Dr. Jacobberger (32). These cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

**Animal studies.** Athymic nude mice were purchased from the Jackson Laboratory and housed in the animal facility at the University of Maryland School of Medicine. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC # 0613011) and conducted following university’s animal policy in accordance with NIH guidelines.

**Prostate tumor samples.** A total of 194 prostate cancer specimens were obtained from the Vancouver Prostate Tissue Bank at the University of British Columbia (Clinical Research Ethics Board number: H09-01628). All specimens were from radical prostatectomy except for 12 CRPC samples, which were obtained from transurethral resections of prostate tumor tissue. H&E slides were reviewed by a pathologist and relevant areas marked. The TMA was manually constructed by punching duplicate 1 mm cores from each sample.

**Plasmids, cloning, and mutagenesis.** The human AKR1C3 construct was obtained by PCR using Rv1 cDNA as template and cloned into pcDNA-Flag, pcDNA-Myc or pET-15b vector. The catalytically-inactive mutation (Y55F) on AKR1C3 was generated using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies). Wild-type or catalytically-inactive mutant AKR1C3 (Y55F) was also subcloned into the pLvx-IRES-zsGreen1 vector.
AR, NCOR1, or Siah2 (N-terminal, middle or C-terminal) fragments in pcDNA-Flag vector and Siah2 in pEGFPN1 or pGEX-4T-2 vectors were described previously (19). Primers used for cloning are available upon request.

**Immunohistochemistry.** Section of the human prostate cancer TMA was used for AKR1C3 IHC. Antigen retrieval was performed using Dako target retrieval solution, followed by peroxidase blocking for 30 min with 3% hydrogen peroxide. Specimens were incubated with AKR1C3 mouse antibody (Sigma) diluted in Dako antibody diluent (1:500) overnight at 4°C. Slides were then washed three times with PBS/Tween-20 and incubated with Dako-Labelled Polymer-HRP (anti-mouse) for 1 h at RT. Slides were then washed 4 times with PBS/Tween-20, developed with DAB, and counterstained with hematoxylin. TMA slides were scanned using a BLISS digital imaging system (Olympus Canada Inc., Richmond Hill, Ontario). Images were viewed using digital Image Hub (SlidePath digital pathology solution, Dublin, Ireland). To quantify AKR1C3 staining, staining intensity was classified as: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). 0 and 1 were defined as low expression, and 2 and 3 were defined as high expression. Staining and quantification of Siah2 were described previously (19). To compare Siah2 and AKR1C3 staining, the original Siah2 quantification was transformed as follows: 0 (1-20 positively-stained cells), 1 (21-50 positively-stained cells), 2 (51-100 positively-stained cells), and 3 (>100 positively-stained cells).

**Lentiviral vector packaging and prostate cancer cell transduction.** The lentiviral vector (shSiah2, shAKR1C3, WT or mutant AKR1C3) was packaged in 293T cells using calcium phosphate transfection. Supernatants containing lentiviral particles were collected 48 h later. PCa cells (Rv1, LNCaP, PC3 or DU145) were transduced with supernatants in the presence of polybrene (8 μg/ml) for 24 h before replacement of media with fresh growth media. Cells were analyzed at 48 h post-transduction. Cells were harvested for analysis at 24 h or 48 h post-transfection.

**Rv1 prostate tumor model.** 8-week-old male nude mice were anesthetized using pentobarbital (90 mg/kg body weight, i.p.). The lower abdomen was opened and the prostate identified under a dissection microscope. For the orthotopic tumor model, Rv1 cells (5 x 10^5 cells/30 μl of 1:1 PBS:Matrigel) transduced with indicated lentiviral vectors were injected into the dorsal prostate using a 30 G needle to achieve ballooning of the prostate, which ensured intraprostatic injection (n = 6 mice/group). Buprenorphine (0.1mg/kg body weight, s.c.) served as postoperative analgesic. All animals were viable after the procedure and recovered readily. Three weeks later, animals were euthanized, prostate tumors were dissected, and prostate tumor weight was determined. For the subcutaneous tumor model, Rv1 cells (2x10^6 cells/100 μl of 1:1 PBS:Matrigel) transduced with control or shSiah2 lentiviral vectors were subcutaneously injected into the flanks of male nude mice (n=6 mice/group). After two weeks, half mice from each group were castrated, while the other half were sham-castrated. Two weeks later, mice were euthanized and tumors were collected for protein and RNA analyses.

**Determination of cellular testosterone and dihydrotestosterone levels.** Relevant Rv1 cells were maintained in media containing 5% CS-FBS for 3 days. Cell lysates were collected using RIPA buffer. Equal amounts of cell lysates were subjected to ELISA assays using T or DHT ELISA kits following the manufacturer’s instruction (Diagnostics Biochem, Dorchester, Canada).

**Soft agar assay.** Cells (5x10^4) were mixed with agar to a final concentration of 0.4% and layered on top of 0.8% agar in 6-well plates. Duplicate plates were incubated at 37°C for 3 weeks. Colony number was determined after staining with p-iodonitrotetrazolium violet. For quantification, colonies with diameters >50 μm were scored in 10 high-power fields.

**MTT cell proliferation assay.** Cells (5x10^3) were seeded into 96-well plates in triplicate. 24 h later, MTT solution (Sigma) was added to each well and cells were incubated for 3 h and the formazan dye formed was then solubilized with acidic isopropanol. Absorbance at 570 nm was
measured using a synergy HT microplate reader (BioTek) and background absorbance at 630 nm was subtracted.

**Immunoprecipitation and western blotting.** For immunoprecipitation, cells were harvested in the IP lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM sodium orthovanadate, and a 1x protease inhibitor cocktail. To immunoprecipitate Flag-tagged proteins, lysates were incubated with M2 beads (Sigma) overnight, beads were washed three times, and precipitated proteins were eluted in SDS loading buffer. To immunoprecipitate endogenous proteins, lysates were incubated with 5 µg of primary antibodies overnight followed by incubation with Protein A/G beads (Santa Cruz) for 4 h. After three washes, precipitated proteins were eluted with SDS loading buffer. For whole cell lysates, cells were harvested using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, and 1x protease inhibitor cocktail). Lysates were subjected to SDS-PAGE and proteins transferred to a nitrocellulose membrane (GE Healthcare Life Sciences). The membrane was probed with primary antibodies followed by secondary antibody conjugated to fluorescent dye, and blots were imaged and quantified using the Odyssey detection system (LI-COR Biotechnology).

**In vitro binding assay.** GST-Siah2 or His-AKR1C3 was expressed in E.coli (BL21) and then purified using glutathione resin (Clontech) or Ni-NTA agarose (Invitrogen), respectively, following manufacturers’ protocols. For in vitro binding, GST-Siah2 and His-AKR1C3 (0.5 µg each) in 0.5 ml of IP lysis buffer were rotated overnight followed by incubation with glutathione resin at 4°C for 1 h. After three washes, proteins bound on beads were eluted with SDS loading buffer and subjected to western blot analysis.

**In vitro ubiquitination assay.** Recombinant E1, E2 and ubiquitin were purchased from Boston Biochem (Cambridge, MA). For the ubiquitination reaction (30 µl), GST-Siah2 (0.5 µg), E1 (60 ng), E2 (UbCHS, 250 ng), ubiquitin (1 µg) and increasing amount of His-AKR1C3 were mixed in buffer containing 50 mM Tris-HCl, pH8.0, 5 mM MgCl2, 0.5 M DTT, 2 mM NaF and 2 mM ATP, and incubated at 37°C for 45 min. Samples were diluted in 0.5 ml IP lysis buffer and incubated with glutathione resin at 4°C for 1 h. After three washes, GST-Siah2 bound to beads was eluted with SDS loading buffer and used for western blot analysis.

**Chromatin immunoprecipitation (ChIP) assay.** Assays were conducted in triplicate. Briefly, cells were crosslinked using 1% formaldehyde for 10 min at RT and then quenched with 5 M glycine. Cells were then lysed and sonicated to obtain 500-bp chromatin fragments. 100 µg of chromatin was incubated with 5 µg antibodies overnight at 4°C followed by incubation with 30 µl protein A/G beads for 4 h. After four washes, crosslinking was reversed, and DNA was purified using spin columns and subjected to qPCR analysis. PCR primers targeting androgen response elements (ARE) in the PSA enhancer were described previously (19).

**qRT-PCR analysis.** Total cellular RNA was prepared using a total RNA miniprep kit (Sigma) and treated with DNase I. cDNA was synthesized using random hexamers for SYBR Green qPCR analysis. A cyclophilin (CPH) primer served as internal control. Triplicate or duplicate samples were used for qPCR analysis. Data was reported as means ± SD. PCR primers were designed using Primer3 or synthesized according to the PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html). Primers for qPCR analysis of human AKR1C3 transcripts were: 5’-GTCAATCGTATTCTAACCGGAG-3’ and 5’-CC ACCCATCGTTTGTCCTGTT-3’. Other primers used were described previously (19).

**Statistical analysis.** Data were analyzed using Student’s t-test. P < 0.05 was considered statistically significant.

**Results**

*Siah2 promotes AKR1C3 expression in certain prostate cancer cells*

Our previous microarray analysis revealed reduced AKR1C3 mRNA levels in Siah2-knockdown (Siah2-KD) Rv1 cells (19). To confirm that Siah2 regulates AKR1C3 protein levels, we knocked down Siah2 in several PCa cell lines, including AR-positive Rv1 and LNCaP cells and AR-negative PC3 and DU145
cells. Cells were maintained in growth media containing charcoal stripped FBS (CS-FBS) or CS-FBS plus the synthetic androgen R1881, to determine the effect of Siah2 on AKR1C3 expression under low-androgen or normal conditions, respectively. CS-FBS contains very low levels of androgens compared with FBS and is widely used to mimic castration conditions in vitro. Western blot analyses showed that Siah2 knockdown (KD) reduced AKR1C3 levels in Rv1 and DU145 cells, either with or without R1881 treatment, but did not alter AKR1C3 levels in PC3 cells (Figure 1A). AKR1C3 protein levels in LNCaP cells were undetectable under both conditions tested by western blot (Figure 1A). Consistent with this, qRT-PCR analyses showed that basal AKR1C3 transcript levels were 25 to 50-fold lower in LNCaP than in other PCa cells tested (namely, Rv1, PC3, or DU145) (Figure 1B). To examine the effect of Siah2 on AKR1C3 expression in vivo, we subcutaneously injected Rv1 cells (pLKO.1 or Siah2-KD) into the nude mice to allow tumor formation, and then castrated the mice for 2 weeks. Western blot analysis showed that Siah2 KD reduced AKR1C3 levels in the Rv1 tumor tissues from both castrated and control mice (Figure 1C), indicating that Siah2 promotes the expression of AKR1C3 protein under both castration and normal conditions.

To determine whether Siah2 regulates AKR1C3 transcription, we performed qRT-PCR analyses of AKR1C3 in prostate cancer cell lines upon Siah2 KD. Of note, R1881 treatment increased PSA transcript levels but decreased AKR1C3 transcripts by ~1.5 fold in Rv1 cells (Figure 1D), consistent with other reports that PSA and AKR1C3 transcripts are androgen-stimulated and androgen-repressed, respectively (21,33). Irrespective of this differential regulation of PSA and AKR1C3 by androgens, in Siah2-KD Rv1 cells both AKR1C3 and PSA transcript levels were reduced independent of whether or not cells are treated with exogenous R1881 (Figure 1D). Similar to these in vitro results, castration of mice decreased PSA transcripts but increased AKR1C3 transcripts in the Rv1 xenograft tumor (Figure 1E); Siah2 KD reduced transcript levels of PSA and AKR1C3 in the Rv1 tumor tissues from both castrated and control mice (Figure 1E). Thus, these results indicate that Siah2 promotes AKR1C3 transcription under both castration and normal conditions. In contrast to Rv1 cells, AKR1C3 transcript levels in DU145 and PC3 cells were not regulated by exogenous androgen (Figure 1F, 1G). Interestingly, however, Siah2 KD in DU145 cells (Figure 1F) but not in PC3 cells (Figure 1G) reduced AKR1C3 transcript levels. Because Siah2 regulates AR activity, and AKR1C3 transcripts are repressed by androgen, we also knocked down AR in Rv1 cells and evaluated potential effects on AKR1C3 transcript levels. AR KD reduced PSA transcript levels but had little effect on AKR1C3 transcripts in the presence or absence of R1881 (Figure 1H), suggesting that AR does not regulate AKR1C3 transcription in the Rv1 line. These results, together with the observation that Siah2 regulates AKR1C3 transcripts in AR-negative DU145 cells, suggest that Siah2 promotes AKR1C3 transcription independent of AR in certain PCa cell lines.

**AKR1C3 is a downstream effector of Siah2 in Rv1 cells**

Previously, we reported that Siah2 activity promotes growth of AR-positive Rv1 cells but has little effect on AR-negative DU145 cells (19). Therefore, we conducted functional investigations of AKR1C3 using Rv1 cells as a model. To do this, we re-expressed AKR1C3 in Siah2-KD Rv1 cells using a lentiviral vector encoding AKR1C3. We have optimized lentiviral transduction conditions to restore AKR1C3 levels in Siah2-KD cells to levels seen in pLKO.1 control cells (Figure 2A). Based on MTT assays, we found that Siah2 KD blocked proliferation of Rv1 cells grown in media containing either FBS or CS-FBS over a 4-day period (Figure 2B, 2C). AKR1C3 re-expression in Siah2-KD cells partially rescued this proliferation defect in Siah2 KD cells (Figure 2B, 2C). Siah2-KD Rv1 cells also showed 5- to 6-fold reduction in colony formation in a soft agar assay, whereas AKR1C3 re-expression partially rescued colony formation in these cells (Figure 2D).

To further test AKR1C3 function in Siah2-dependent tumorigenesis, we used an orthotopic prostate tumor model in which Rv1 cells are injected into the prostate of nude mice.
Injection of control (pLKO.1) Rv1 cells resulted in formation of large prostate tumors, whereas injection of Siah2-KD (shSiah2) Rv1 cells under comparable conditions promoted a ~10-fold reduction in the relative weight of tumors formed (Figure 2E). AKR1C3 re-expression in Siah2-KD cells (shSiah2+AKR1C3) resulted in a 5-fold increase in tumor weight relative to tumors formed in Siah2-KD cells (Figure 2E).

Next, we performed immunohistochemistry staining for the proliferation marker Ki67 and active caspase-3, a marker of apoptosis, on tumor sections. Siah2 KD reduced the percentage of Ki67-positive cells, concomitant with an increased percentage of cells positive for active caspase-3 (Figure 2F, 2G), suggesting that Siah2 inhibits proliferation and promotes apoptosis in orthotopic prostate tumors. In contrast, patterns of Ki67 and active caspase-3 staining seen in Siah2-KD tumors were partially reversed after AKR1C3 re-expression (Figure 2F, 2G). Overall, these findings confirm that AKR1C3 functions as a downstream effector of Siah2 in driving PCa growth in vitro and in vivo.

**AKR1C3 re-expression partially restores AR activity in Siah2-KD Rv1 cells**

Since AKR1C3 re-expression partially rescued growth of Siah2-KD Rv1 cells (Figure 2), we asked whether AKR1C3 regulates AR activity. As we previously reported, Siah2 KD in Rv1 cells reduced transcript levels of specific AR target genes, such as PSA, NKX3.1 and PMEPA1 (Figure 3A), which we interpret as reflecting downregulated AR transcriptional activity. In contrast, AKR1C3 re-expression in Siah2-KD Rv1 cells partially increased transcript levels of these AR target genes (Figure 3A). We previously reported that Siah2 ubiquitinated and targeted for degradation a transcriptionally inactive AR/NCOR1 complex on the PSA gene enhancer, thus allowing for AR/co-activator complexes to bind to and mediate PSA gene transcription (19). To determine whether AKR1C3 regulates AR/NCOR1 complex on the PSA gene, we performed a chromatin-immunoprecipitation (ChIP) assay using either AR, NCOR1 or acetylated histone H3 (acetyl-H3) antibody in pLKO.1 control, Siah2-KD, or Siah2-KD + AKR1C3 Rv1 cells. As expected, Siah2 KD increased levels of NCOR1-bound AR on the PSA enhancer and decreased acetylation of histone H3, an epigenetic mark of transcriptionally active chromatin (Figure 3B).

In contrast, AKR1C3 re-expression in Siah2-KD cells partially reversed levels of both the AR/NCOR1 complex and acetylated histone H3 (Figure 3B), suggesting that AKR1C3 functions in Siah2-dependent removal of AR/NCOR1 complex from the PSA enhancer.

**AKR1C3 alone cannot rescue androgen levels decreased by Siah2 KD in Rv1 cells**

AKR1C3 has been implicated in the androgen-independent growth of PCa cells via upregulation of intratumoral androgen biosynthesis (27). Thus we asked whether Siah2 controls androgen biosynthesis through AKR1C3 and, if so, whether this pathway regulates AR activity. To test this, we employed ELISA assays to measure T and DHT levels in lysates of pLKO.1 control, Siah2-KD, or Siah2-KD + AKR1C3 Rv1 cells grown in CS-FBS media for 3 days. Siah2 KD reduced T and DHT levels relative to controls by ~30% (Figure 3C, 3D), but AKR1C3 re-expression failed to rescue these phenotypes (Figure 3C, 3D), possibly because Siah2 KD deregulates additional steroidogenic enzymes.

To further investigate whether lower androgen levels seen in Siah2-KD cells contribute to reduced AR activity, we added exogenous androgens to Siah2-KD Rv1 cells and assessed potential rescue of reduced PSA transcript levels. qRT-PCR analysis revealed that addition of 1 nM R1881 or 10 nM DHT slightly increased PSA transcript levels in both control pLKO.1 and Siah2-KD cells, but did not rescue PSA transcript levels in Siah2-KD cells to basal levels seen in control cells without androgen treatment (Figure 3E). Similarly, in MTT cell proliferation assays, addition of 1 nM R1881 or 10 nM DHT did not rescue proliferation defects seen in Siah2-KD Rv1 cells over a 3-day period (Figure 3F). Overall, these results demonstrate that the small reduction of androgen levels (~30%) seen in Siah2-KD Rv1 cells is insufficient to cause a decrease in PSA transcript levels or in cell proliferation. Thus, the 30% reduction in androgen levels does not appear to impair AR activity among such cells.
To assess AKR1C3 biochemical function in Siah2-dependent cell proliferation, we generated a catalytically inactive (CI) mutant form of AKR1C3 with a point mutation of Tyrosine 55 residue (Y55F) in the catalytic triad (34,35). We first confirmed that the Y55F mutation indeed resulted in the loss of catalytic activity of AKR1C3 because overexpression of wild-type but not the mutant AKR1C3 in Rv1 cells increased the intracellular levels of T or DHT (Figure 4G, 4H). We then expressed either the mutant or wild-type AKR1C3 in Siah2-KD Rv1 cells in order to assess the effect on cell proliferation. Western blot analysis confirmed re-expression of mutant or wild-type forms of AKR1C3 in Siah2-KD cells to endogenous levels seen in pLKO.1 control cells (Figure 3I). Based on MTT (Figure 3J) or soft agar (Figure 3K) assays, respectively, re-expression of mutant AKR1C3 in the Siah2-KD Rv1 cells partially rescued growth or colony formation to a similar extent as did expression of wild-type AKR1C3, indicating that AKR1C3 functions in Siah2-dependent regulation of cell proliferation independent of its catalytic activity.

**AKR1C3 enhances Siah2 stability and protein levels**

We next asked how AKR1C3 promotes Siah2-dependent AR activity and cell proliferation. Interestingly, we observed a partial increase in the endogenous Siah2 protein level upon re-expression of either the WT or CI mutant form of AKR1C3 in Siah2-KD Rv1 cells (Figure 3I), without changes in Siah2 transcript levels (Figure 3L). Overexpression of Flag-AKR1C3 in 293T cells also increased protein levels of overexpressed GFP-Siah2 (Figure 4A). Furthermore, in Rv1 cells, Flag-AKR1C3 overexpression (Figure 4B) increased while AKR1C3 KD (Figure 4D) decreased endogenous Siah2 protein levels, but neither manipulation altered Siah2 transcript levels (Figure 4C, 4E). We also knocked down AKR1C3 in DU145 and PC3 cells using two different AKR1C3 shRNAs and in both cases observed 40-50% reduction in Siah2 protein levels (Figure 4F), whereas no changes in Siah2 transcripts occurred (Figure 4G). Overall, these findings show that AKR1C3 enhances expression of Siah2 protein rather than mRNA.

To further examine the relationship between Siah2 and AKR1C3, we co-expressed Flag-AKR1C3 with either WT GFP-Siah2 or the GFP-Siah2 RING mutant (which lacks ubiquitin ligase activity) in 293T cells. Over-expression of either WT or RING mutant Siah2 had no effect on Flag-AKR1C3 protein levels (Figure 4H, lane 3 or lane 4 vs lane 6), indicating that Siah2 activity does not alter AKR1C3 protein levels. As previously reported (13,36,37), levels of WT Siah2 protein are very low compared with that of Siah2 RING mutant (Figure 4H, lane 2 vs lane 5). Expression of Flag-AKR1C3 increased protein levels of WT Siah2 by 3- to 4-fold (Figure 4H, lane 3 vs lane 2) but had little effect on levels of RING mutant Siah2 (Figure 4H, lane 4 vs lane 5), suggesting that AKR1C3 inhibits Siah2 ubiquitin ligase activity and hence self-ubiquitination. To test this possibility, we performed a cycloheximide chase experiment to determine GFP-Siah2 half-life in the presence of co-expressed Flag-AKR1C3 in 293T cells. GFP-Siah2 half-life in the absence or presence of Flag-AKR1C3 was ~1 or ~2 hours, respectively (Figure 4I), indicating that AKR1C3 increases Siah2 stability. To determine if AKR1C3 catalytic activity is required for this effect, we co-expressed GFP-Siah2 with Myc-AKR1C3 (WT or CI mutant) in 293T cells. The presence of CI AKR1C3 increased Siah2 protein levels in a manner comparable to WT AKR1C3 (Figure 4J), indicating that the effect of AKR1C3 on the Siah2 protein level is independent of AKR1C3’s catalytic activity. Finally, to determine the effect of AKR1C3 on the Siah2 stability in Rv1 cells, we performed the cycloheximide chase experiment to determine the degradation kinetics of endogenous Siah2 upon co-expression of Flag-AKR1C3. The half-life of endogenous Siah2 in the absence or presence of Flag-AKR1C3 was approximately 1 or 2 hours, respectively (Figure 4K). These results indicate that co-expression of Flag-AKR1C3 increased the stability of endogenous Siah2 in Rv1 cells.

**AKR1C3 blocks Siah2 self-ubiquitination and degradation**

The above findings suggest that AKR1C3 and Siah2 may interact physically. To test this possibility, we performed immunoprecipitation analysis in 293T cells co-expressing Flag-Siah2
and Myc-AKR1C3. When we precipitated Flag-Siah2 with anti-Flag M2 beads, we observed co-precipitated Myc-AKR1C3 (Figure 5A), indicative of interaction. To determine if the interaction was direct, we purified recombinant GST-Siah2 and His-AKR1C3 proteins from *E. coli* and performed an in vitro binding assay. Western blotting analysis showed that His-AKR1C3 was pulled down by Siah2-bound beads but not by beads alone (Figure 5B), indicating that the interaction is direct. Siah2 consists of an N-terminal region, a central RING Finger/Zinc Finger domain, and a C-terminal substrate-binding domain. To determine which domain(s) interacts with AKR1C3, we co-expressed Myc-AKR1C3 and Flag-tagged Siah2 fragments (N-terminal, middle or C-terminal) in 293T cells and performed immunoprecipitation using anti-Flag beads. Western blot analysis showed that Myc-AKR1C3 was co-precipitated by both the N-terminal and C-terminal regions of Siah2 (Figure 5C).

To determine whether Siah2/AKR1C3 interaction alters Siah2 self-ubiquitination, we co-expressed Flag-Siah2, HA-ubiquitin (Ub) and Myc-AKR1C3 in 293T cells, treated cells with the proteasome inhibitor MG132 for 4 h, and then precipitated Flag-Siah2 using anti-Flag M2 beads and performed western blotting analysis with HA antibody to detect ubiquitination of Flag-Siah2. As expected, Siah2 was ubiquitinated in the absence of Myc-AKR1C3 (Figure 5D, lane 2), as indicated by smears of high molecular weight proteins at the interface of the stacking and separating gels. Co-expression of Myc-AKR1C3, however, significantly reduced Siah2 ubiquitination (Figure 5D, lane3). Of note, Siah2 protein levels in the presence or absence of AKR1C3 were comparable in MG132-treated cells (Figure 5D), in contrast to levels seen in cells not treated with MG132 (Figure 4A, 4H-4J). As controls, no polyubiquitinated proteins were co-precipitated with anti-Flag M2 beads in cells expressing the HA-ubiquitin alone (Figure 5D, lane 4), and the expression of HA-ubiquitin was similar in the input samples (Figure 5D, lane 2 to 4). These data support our hypothesis that AKR1C3 increases Siah2 protein levels by inhibiting self-ubiquitination and hence stabilizing Siah2.

To directly test if AKR1C3 inhibits Siah2 self-ubiquitination, we conducted an in vitro self-ubiquitination assay using ubiquitin, E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and GST-Siah2, with or without His-AKR1C3, followed by GST-Siah2 pull-down by glutathione beads and western blotting with ubiquitin (Figure 5E, upper panel) or GST (Figure 5E, middle panel) antibodies. We found that GST-Siah2 was self-ubiquitinated in the absence of His-AKR1C3, while GST-Siah2 self-ubiquitination decreased as levels of His-AKR1C3 increased (Figure 5E, upper and middle panels).

Interaction with AKR1C3 apparently blocks Siah2 self-ubiquitination. Then, we asked whether AKR1C3 alters Siah2-dependent degradation of substrates such as AR and NCOR1. To determine this, we co-expressed GFP-Siah2 plus either Flag-AR or Flag-NCOR1 with Flag-AKR1C3. GFP-Siah2 overexpression alone decreased levels of both Flag-AR (Figure 5F) and Flag-NCOR1 (Figure 5G), as reported (19,38). Flag-AKR1C3 over-expression increased Siah2 protein levels and potentiated Siah2-dependent decreases in Flag-AR (Figure 5F) or Flag-NCOR1 (Figure 5G) levels, indicating that increased Siah2 protein levels seen following AKR1C3 overexpression enhance degradation of Siah2 targets. Taken together, the above studies demonstrate that AKR1C3 interacts directly with Siah2 and inhibits its self-ubiquitination and degradation but does not inhibit Siah2’s degrading function of other targets.

**AKR1C3 protein levels parallel those of Siah2 in a prostate cancer TMA and correlate with poor prognosis**

To assess the relevance of our findings to human PCa, we evaluated both AKR1C3 and Siah2 protein expression by immunohistochemistry in a PCa tissue microarray (TMA) containing representative samples of different Gleason grades as well as CRPC. As previously reported (25), AKR1C3 staining was largely cytoplasmic, but nuclear staining of AKR1C3 was also observed in some prostate cancer cells (Figure 6A). Consistent with Siah2 self-ubiquitination/degradation activity, Siah2 staining was detectable only in...
the nuclei of some prostate cancer cells (Figure 6A). This staining pattern of Siah2 is consistent with the previous reports of Siah2 staining in cancers (14,39,40). Quantification of AKR1C3 and Siah2 staining revealed a similar pattern in this cohort of PCa samples: both were weakly expressed in Benign Prostate Hyperplasia (BPH), and in general increased in PCa with increasing Gleason scores and in CRPC (Figure 6B, 6C).

To determine whether AKR1C3 and Siah2 levels correlate with poor prognosis, we divided specimens into AKR1C3-low or AKR1C3-high groups. Similarly, specimens were classified as Siah2-low and Siah2-high groups. In these classifications, low expression is defined as weak (score 1) or no staining (score 0), while high expression indicates moderate (score 2) or strong staining (score 3). We then performed Kaplan-Meier curve analysis to determine whether AKR1C3 and Siah2 expression levels were associated with biochemical recurrence based on PSA analysis, after surgery or radiation therapy. The average time to biochemical recurrence was 101 and 57 months for AKR1C3-low and AKR1C3-high groups, respectively, and 101 and 82 months for Siah2-low and Siah2-high groups, respectively (Figure 6D, 6E). Thus, high levels of both AKR1C3 and Siah2 are associated with more rapid prostate cancer recurrence after definitive locally directed therapies for prostate cancer.

Discussion

We previously established a role for the ubiquitin ligase Siah2 in promoting AR transcriptional activity by regulating turnover of an AR/NCOR1 complex on specific AR target genes (19). One Siah2-regulated gene is AKR1C3, which encodes a steroidogenic enzyme implicated in intratumoral androgen biosynthesis, resistance of PCa cells to AR antagonists, and PCa progression (20,21,24-27). Therefore, we initially asked whether Siah2-dependent AKR1C3 transcription promoted androgen biosynthesis in PCa cells and, if so, whether this activity functions in Siah2-dependent AR activity.

We confirmed that Siah2 inhibition decreased AKR1C3 expression and slightly lowered intracellular androgen levels in AR-positive Rv1 prostate cancer cells. This result is consistent with reports that Rv1 cells can synthesize androgen (41,42) and with our profiling array data revealing regulation of steroidogenic enzymes by Siah2 (19). Restoring the expression of AKR1C3 in the Siah2-KD Rv1 cells partly rescued the AR activity and cell proliferation defects, demonstrating the AKR1C3 is a key downstream effector of Siah2. Although AKR1C3 is critical for androgen biosynthesis in tumors, its re-expression alone in Siah2-KD Rv1 cells did not rescue intracellular androgen levels, suggesting that AKR1C3 plus other Siah2-regulated steroidogenic enzymes (e.g. HSD17B8, HSD17B14) may be required to restore the androgen biosynthesis in the Siah2-KD Rv1 cells. Notably, although androgen levels were lowered by ~30% in Siah2-KD Rv1 cells, addition of exogenous androgens did not raise levels of PSA transcripts in Siah2-KD cells to comparable levels in control cells nor did it rescue growth defects seen in Siah2-KD cells. These results indicate that a moderate 30% change in intracellular androgen level is insufficient to alter the AR activity and cell proliferation in Rv1 cells. Furthermore, re-expression of a catalytically inactive mutant AKR1C3 showed a similar rescue effect on the growth of Siah2-KD Rv1 cells as that of wild-type AKR1C3. Thus, we conclude that AKR1C3 contributes to Siah2-dependent AR activity and cell proliferation independent of its role in the intratumoral androgen biosynthesis.

We previously reported that Siah2 protein levels are upregulated in PCa tissues, compared with the benign prostate hyperplasia (19), but it remained unclear how Siah2 proteins were upregulated in these cancers. According to the Oncomine database of published microarray data, Siah2 mRNA levels are largely unchanged in PCa compared with normal prostate tissue. Only small sets of metastatic PCa or CRPC showed a moderate 1.5- to 2-fold increase in Siah2 mRNA (18,19). These observations suggest that alteration in Siah2 transcription may not be the primary mechanism underlying increased Siah2 activity observed in PCa tissues. As an E3 ubiquitin ligase, Siah2 regulates its own stability via self-ubiquitination and subsequent degradation (13,36,43). In this study, we found that AKR1C3 directly interacts with
Siah2 to inhibit its self-ubiquitination, thus increasing Siah2 protein levels and hence activity in PCa cells. Consistent with this notion, AKR1C3 knockdown in PCa cells (Rv1, PC3, DU145) reduced Siah2 protein levels without altering Siah2 mRNA expression. We conclude that in some prostate cancer cells such as Rv1 and DU145 cells, Siah2 promotes AKR1C3 transcription, while AKR1C3 increases Siah2 stability, thereby potentially forming a positive regulatory loop between these factors. Importantly, our analysis of a prostate cancer TMA indicated that expressions of Siah2 and AKR1C3 change in concert and tend to correlate positively with increasing Gleason grade. Further, higher expressions of Siah2 or AKR1C3 are associated with biochemical recurrence after locally directed therapies (prostatectomy, radiation), and in patients with CRPC.

Although Siah2 is required for AKR1C3 expression in certain PCa cells, it remains to be determined how Siah2 promotes AKR1C3 transcription. We previously found that Siah2 promoted the transcription of select AR target genes by turnover of transcriptionally inactive AR/NCOR1 complex on these genes (19). However, this mechanism does not apply to the Siah2-dependent transcription of AKR1C3, which appears to be independent of AR. (i) Siah2 is required for AKR1C3 transcription in both AR-positive Rv1 cells and AR-negative DU145 cells; (ii) Siah2 regulates the basal transcription of AKR1C3 independent of androgens; (iii) AR knockdown in Rv1 cells does not affect AKR1C3 transcript levels. Thus, the transcription of AKR1C3 is Siah2-dependent but AR-independent. In addition to AR and NCOR1, Siah2 has been shown to directly destabilize other transcription factors or co-factors in a cell type- and context-dependent manner—among them are PPARγ, Nrf2, C/EBPδ, and HDAC3 (44-47). Siah2 can also indirectly regulate activity of some transcription factors (such as HIF-1α, NF-kB, STAT3, and YAP) by promoting degradation of their upstream regulators (48-51). Future work is needed to determine which transcription factor(s) regulated by Siah2 may be required for basal AKR1C3 expression in PCa cells.

In addition to AR signaling, Siah2 has been shown to regulate several other signaling pathways (such as hypoxia signaling and MAP kinase signaling, among others) that are important for cancer development and progression (10,15,16,18). We demonstrate here that the mutual regulation between Siah2 and AKR1C3 promotes the Siah2-dependent regulation of AR signaling in the AR-positive Rv1 cells. Interestingly, the Siah2-AKR1C3 regulation loop is also observed in the AR-negative DU145 cells. It will be interesting to investigate whether the Siah2-AKR1C3 axis promotes some of the AR-independent signaling pathways that may also be operative in prostate cancer, and perhaps other cancer types as well.

Our study using an Rv1 prostate cancer cell model reveals a catalytically-independent role of AKR1C3 in controlling Siah2 stability and thus regulating Siah2-dependent AR activity. Notably, it was recently reported that AKR1C3 (WT or C1 mutant) could function as an AR co-activator (52), another example of a catalytically-independent role of AKR1C3 on AR activity. Therefore, identification of mechanisms underlying the non-catalytic function of AKR1C3 may provide new targets for development of novel AKR1C3 inhibitors that complement inhibitors targeting AKR1C3 catalytic activity as potential CRPC therapy.
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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contribution

JQ conceived and coordinated the study and wrote the paper. LF and GP designed, performed and analyzed the experiments shown in Figure 1 to Figure 5. FL, EG and MG designed, performed and analyzed the experiments shown in Figure 6. AH provided technical assistance and contributed to the preparation of the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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Abbreviations: AKR1C3, aldo-keto reductase family 1, member C3; Siah2, siah E3 ubiquitin protein ligase 2; AR, androgen receptor; CRPC, castration-resistant prostate cancer; ADT, androgen deprivation therapy; T, testosterone; DHT, dihydrotestosterone.
Figure Legends

Figure 1

A. Effect of Siah2 KD on AKR1C3 protein levels. Indicated PCa cell lines were transduced with pLKO.1 (#1) or shSiah2 (#2) and maintained in media containing 5% CS-FBS for 48 h. Cells were either treated or not treated with 1 nM R1881 for 24 h and analyzed by western blotting with antibodies to AKR1C3 and tubulin, which served as a loading control. B. Effect of exogenous androgen (R1881) on AKR1C3 transcript levels in PCa cells. Indicated cells were maintained in media containing 5% CS-FBS for 48 h, and then either treated or not treated with 1nM R1881 for 24 h. Cells were analyzed for AKR1C3 transcripts by qRT-PCR. R1881 treatment reduced AKR1C3 transcript levels in LNCaP and Rv1 cells (p<0.05) but not in PC3 or DU145 cells (p>0.1). C. Effect of Siah2 KD on AKR1C3 protein levels in xenograft tumors. Nude mice harboring Rv1 xenograft tumors (pLKO.1 or shSiah2) were either castrated or sham-castrated for 2 weeks. Tumor tissues (n=3/group) were analyzed by western blotting with AKR1C3 or tubulin antibodies. D. Effect of Siah2 KD on AKR1C3 transcript levels in Rv1 cells. Cells were treated and analyzed as in B. Siah2 KD in Rv1 cells reduced AKR1C3 and PSA transcript levels without (p<0.01 for Siah2 or PSA, p<0.005 for AKR1C3) or with (p<0.005 for Siah2 or AKR1C3, p<0.05 for PSA) R1881 treatment. E. Effect of Siah2 KD on AKR1C3 transcripts in xenograft tumors. The xenograft tumors described in C were analyzed by qRT-PCR for Siah2, AKR1C3 or PSA transcripts. Siah2 KD reduced AKR1C3 and PSA transcript levels in control (p<0.005 for AKR1C3, p<0.001 for PSA or Siah2) or castrated (p<0.005 for Siah2 or AKR1C3, p<0.05 for PSA) mice. F and G. Effect of Siah2 KD on AKR1C3 transcript levels in DU145 (F) and PC3 cells (G). Indicated PCa cells were treated and analyzed as in B. Siah2 KD in DU145 cells reduced AKR1C3 transcript levels without (p<0.005 for Siah2 or AKR1C3) or with (p<0.005 for AKR1C3) R1881 treatment. In G, Siah2 KD in PC3 cells had no effect on AKR1C3 transcript levels without (p<0.01 for Siah2, p>0.1 for AKR1C3) or with (p<0.01 for Siah2, p>0.1 for AKR1C3) R1881 treatment. H. Effect of AR KD on AKR1C3 transcript levels. Rv1 cells (pLKO.1 or shAR) were treated as in B and analyzed by qRT-PCR for AR, AKR1C3 or PSA transcripts. AR KD in Rv1 cells reduced PSA transcript levels but not AKR1C3 without (p<0.005 for AR, p<0.05 for PSA, p>0.05 for AKR1C3) or with (p<0.005 for AR or PSA, p>0.05 for AR for AKR1C3) R1881 treatment.

Figure 2

A. AKR1C3 re-expression in Siah2-KD cells. Siah2-KD Rv1 cells were transduced with lentivirus harboring AKR1C3. Cells (control pLKO.1, shSiah2, or shSiah2+AKR1C3) were analyzed by western blotting with AKR1C3 and tubulin antibodies. B and C. Effect of AKR1C3 re-expression on proliferation of Siah2-KD cells. Rv1 cells in A were maintained in media containing either FBS (B) or CS-FBS (C) and assayed for proliferation at indicated time points. Siah2 KD inhibited the growth of Rv1 cells in FBS media (pLKO.1 vs shSiah2: p<5x10^-4 at 24 h, p<10^-5 at 48 h, p<5x10^-6 at 72 h or 96 h) or CS-FBS media (pLKO.1 vs shSiah2: p<5x10^-6 at 24 h or 96 h, p<10^-3 at 48 h, p<10^-2 at 72 h). AKR1C3 re-expression partly promoted growth of Siah2-KD Rv1 cells in FBS media (shSiah2 vs shSiah2+AKR1C3: p<5x10^-4 at 24 h or 72 h, p<5x10^-3 at 48 h, p<5x10^-6 at 96 h) or CS-FBS media (shSiah2 vs shSiah2+AKR1C3: p<5x10^-3 at 24 h or 96 h, p<0.05 at 48 h, p<0.001 at 72 h). D. The effect of AKR1C3 re-expression on colony formation by Siah2-KD cells. Rv1 cells in A were maintained in soft agar for 3 weeks and colony number per-field was determined. The colony formation was reduced upon Siah2 KD (pLKO.1 vs shSiah2: p<5x10^-6), but partly increased upon re-expression of AKR1C3 (shSiah2 vs shSiah2+AKR1C3: p<5x10^-3). E. The effect of AKR1C3 re-expression on orthotopic prostate tumor formation by Siah2-KD cells. Rv1 cells in A were injected into dorsal prostates of nude mice. Three weeks later, tumors were monitored and weighed (n=6 for each group). The tumor weight was decreased upon Siah2 KD (pLKO.1 vs shSiah2: p<5x10^-6), but partly increased upon re-expression of AKR1C3 (shSiah2 vs shSiah2+AKR1C3: p<0.05). F. The effect of AKR1C3 re-expression on proliferation or apoptosis of...
Siah2-KD Rv1 cells in orthotopic prostate tumors. Paraffin sections derived from indicated tumors were analyzed by staining with Ki67 (a proliferation marker) and active caspase-3 (an apoptosis marker). Staining was visualized by DAB (brown) plus a hematoxylin counterstain (blue). G. Quantification of Ki67 and active caspase-3 staining shown in F. The number of positively-stained nuclei and total nuclei was determined in 5 random high-power fields. The percentage of positively-stained cells for Ki67 was reduced upon Siah2 KD (pLKO.1 vs shSiah2: p<0.005), but partly increased upon re-expression of AKR1C3 (shSiah2 vs shSiah2+AKR1C3: p<0.05). The percentage of positively-stained cells for active caspase-3 was increased upon Siah2 KD (pLKO.1 vs shSiah2: p<0.001), but partly decreased upon re-expression of AKR1C3 (shSiah2 vs shSiah2+AKR1C3: p<0.05).

Figure 3

A. Effect of AKR1C3 re-expression in Siah2-KD Rv1 cells on AR target gene expression. Indicated cells were analyzed by qRT-PCR for PSA, NKX3.1 or PMEPA1 transcripts. Siah2 KD reduced the transcript level of these AR targets (pLKO.1 vs shSiah2: p<0.01 for PSA, p<5x10^{-5} for NKX3.1, p<0.005 for PMEPA1). Re-expression of AKR1C3 partly increased these AR targets (shSiah2 vs shSiah2+AKR1C3: p<0.005 for PSA or NKX3.1, p<0.0005 for PMEPA1). B. Effect of AKR1C3 re-expression in Siah2-KD cells on association of AR, NCOR1 or acetylated histone H3 (acetyl-H3) with the PSA enhancer (ARE) based on ChIP analysis using indicated antibodies. Siah2 KD increased the amount of AR and NCOR1, and decreased acetyl-H3 (pLKO.1 vs shSiah2: p<0.05 for AR, p<0.005 for NCOR1 or acetyl-H3). Re-expression of AKR1C3 partly decreased the amount of AR and NCOR1, and increased acetyl-H3 (shSiah2 vs shSiah2+AKR1C3: p<0.05 for AR or acetyl-H3, p<0.01 for NCOR1). C and D. Analysis of intracellular testosterone (T) and dihydrotestosterone (DHT) levels. Rv1 cells were maintained in media containing 5% CS-FBS for 3 days and subjected to ELISA assay for T or DHT. Siah2 KD reduced the level of T or DHT (pLKO.1 vs shSiah2: p<0.05). Re-expression of AKR1C3 in the Siah2-KD Rv1 cells could not increase the level of T or DHT (shSiah2 vs shSiah2+AKR1C3: p>0.1). E. Effect of androgens on PSA transcript levels in Siah2-KD Rv1 cells. Cells were maintained in media containing 5% CS-FBS for 48 h, followed by incubation with DMSO vehicle, 1 nM R1881 or 10 nM DHT for 24 h, and then analyzed by qRT-PCR for PSA transcripts. Siah2 KD reduced the PSA transcript level in any condition (pLKO.1 vs shSiah2: p<0.001 for DMSO or 10 nM DHT, p<0.005 for 1 nM R1881). F. Effect of androgens on proliferation of Siah2-KD Rv1 cells. Cells were maintained in media containing 5% CS-FBS with DMSO vehicle, 1 nM R1881 or 10 nM DHT. Cells were analyzed by an MTT assay at indicated time points. Compared with pLKO.1 control, Siah2 KD reduced cell proliferation at any condition (DMSO: p<5x10^{-4} at 24 h, p<0.001 at 48 h, p<5x10^{-5} at 72h. 1nM R1881: p<5x10^{-3} at 24 h or 48 h, p<5x10^{-4} at 72 h. 10nM DHT: p<5x10^{-5} at 24 h, p<5x10^{-4} at 48 h, p<1x10^{-3} at 72 h). G and H. Effect of AKR1C3 overexpression on the intracellular T (G) or DHT (H). Rv1 cells were transduced with the indicated lentiviral constructs, maintained and analyzed as in C and D. Compared with control, overexpression of WT AKR1C3 increased the levels of T (p<0.01) or DHT (p<0.005), whereas overexpression of mutant AKR1C3 had no effect on either T or DHT (p>0.1). I. Re-expression of wild-type (WT) or catalytically-inactive (CI) mutant AKR1C3 in Siah2-KD Rv1 cells. Siah2 was precipitated from indicated cells using Siah2 antibodies and analyzed by western blotting with Siah2 antibodies. Relative intensity of Siah2 bands was shown at the bottom of the blot. Cell lysates were blotted with AKR1C3 or actin antibodies. J. The effect of re-expression of mutant AKR1C3 on proliferation of Siah2-KD Rv1 cells. Indicated cells were maintained in media containing 5% CS-FBS and analyzed by an MTT assay at indicated time points. Cell proliferation was reduced upon Siah2 KD (pLKO.1 vs shSiah2: p<5x10^{-4} at 24 h, 48 h, or 72 h), but was partly increased upon re-expression of either WT AKR1C3 (shSiah2 vs shSiah2+AKR1C3: p<0.05 at 24 h, p<0.001 at 48 h, p<0.01 at 72 h) or mutant AKR1C3 (shSiah2 vs shSiah2+AKR1C3: p<5x10^{-4} at 24 h, p<0.005 at 48 h or 72 h). K. Re-expression of mutant AKR1C3 on colony formation by Siah2-KD Rv1 cells. Indicated cells were maintained in soft agar for 3 weeks and the number of colonies per-field was determined. The colony formation was reduced upon Siah2 KD (pLKO.1 vs shSiah2: p<5x10^{-15}). Compared with Siah2 KD, re-expression of either WT
AKR1C3 (p<5x10^{-10}) or mutant AKR1C3 (p<1x10^{-10}) partly increased colony formation. L. The effect of AKR1C3 re-expression on Siah2 transcript levels. Indicated Rv1 cells were analyzed by qRT-PCR for Siah2 transcripts. Compared with Siah2 KD, re-expression of WT AKR1C3 (p>0.1) or mutant AKR1C3 (p>0.1) had no effect on the Siah2 transcript level.

**Figure 4**

**A.** Effect of AKR1C3 overexpression on ectopically-expressed Siah2. GFP-Siah2 and Flag-AKR1C3 were co-expressed in 293T cells, and 24 hours later cells were analyzed by western blotting with GFP, Flag, or tubulin antibodies. **B.** Effect of AKR1C3 overexpression on endogenous Siah2 levels. Rv1 cells were transfected with pcDNA control or Flag-AKR1C3 plasmids. After 24 hours, Siah2 was precipitated with Siah2 antibodies and analyzed by western blotting using Siah2 antibodies. Lysates were analyzed by western blotting with Flag or actin antibodies. **C.** Effect of AKR1C3 overexpression on Siah2 transcript levels. Rv1 cells in B were analyzed by qRT-PCR for Siah2 transcripts. Overexpression of Flag-AKR1C3 had no effect on the Siah2 transcript level (p>0.1). **D.** Effect of AKR1C3 KD on Siah2 protein levels. Rv1 cells were transduced with control pLKO.1 or AKR1C3 shRNA and maintained in the media containing 5% CS-FBS for 48 h. Cells were then either treated or not treated with 1 nM R1881 for 24 h. Siah2 was precipitated and analyzed as in B. Relative intensity of Siah2 bands is shown at the bottom of the blot. **E.** Effect of AKR1C3 KD on Siah2 transcript levels. Rv1 cells in D were analyzed by qRT-PCR for Siah2 transcripts. Compared with pLKO.1 control, AKR1C3 KD had no effect on Siah2 transcript levels with (p>0.1) or without (p>0.1) R1881 treatment. **F.** Effect of AKR1C3 KD on Siah2 protein levels in DU145 or PC3 cells. AKR1C3 was knocked down using one of two different shRNAs (sh-1 or sh-2). After 48 h, Siah2 was analyzed as in D. **G.** Effect of AKR1C3 KD on Siah2 transcript levels in DU145 or PC3 cells. Cells in F were analyzed by qRT-PCR for Siah2 transcripts. Compared with pLKO.1 control, knockdown of AKR1C3 with either of the two shRNAs had no effect on the Siah2 transcript level in DU145 (p>0.1) or PC3 (p>0.1) cells. **H.** Effect of AKR1C3 overexpression on levels of Siah2 RING mutant. Flag-AKR1C3 was co-expressed with GFP-Siah2 (WT or RING mutant) in 293T cells, and cells were analyzed by western blotting with GFP, Flag or tubulin antibodies. The ratio between Siah2 and tubulin was shown at the bottom of the blot. **I.** Half-life of ectopically expressed Siah2 following AKR1C3 overexpression. 293T cells were co-transfected with GFP-Siah2 and Flag-AKR1C3, and 24 h later treated with cycloheximide (50 µg/ml). Cell lysates were collected at indicated time points and analyzed by western blotting with GFP, Flag or actin antibodies. The ratio between Siah2 and actin was shown at the bottom of the blot. **J.** Effect of overexpression of the CI mutant AKR1C3 on ectopically expressed Siah2. 293T cells were co-transfected with GFP-Siah2 and Myc-AKR1C3 (WT or CI mutant) and 24 hours later by western blotting with GFP, Myc or tubulin antibodies. **K.** Effect of AKR1C3 overexpression on the stability of endogenous Siah2. Rv1 cells were transfected with Flag-AKR1C3, and treated with cycloheximide as in I. Siah2 was immunoprecipitated from samples at the indicated time points, and analyzed by western blotting with Siah2 antibodies. The relative intensity of Siah2 bands was shown at bottom of the blot. The input samples were blotted with Flag or tubulin antibodies.

**Figure 5**

**A.** Interaction of ectopically expressed Siah2 and AKR1C3. Myc-AKR1C3 and Flag-Siah2 were co-expressed in 293T cells. Flag-Siah2 was precipitated with anti-Flag M2 beads, and bound proteins were analyzed by western blotting with Flag or Myc antibodies. **B.** Siah2/AKR1C3 interaction in vitro. GST-Siah2 and His-AKR1C3 were purified from E. coli and mixed. GST-Siah2 was pulled down using glutathione beads, and bound proteins were analyzed by western blotting with GST or His antibodies. **C.** Interaction of AKR1C3 and Siah2 fragments. 293T cells were transfected with full-length Myc-AKR1C3 and N-terminal (N), middle region (M) or C-terminal (C) fragments of Flag-Siah2. Flag-Siah2 fragments were precipitated with anti-Flag M2 beads, and bound proteins were analyzed by western blotting with Myc or Flag antibodies. Input protein was blotted with Myc antibodies. **D.** Effect of AKR1C3 on Siah2 self-ubiquitination. Flag-Siah2 and HA-ubiquitin (Ub) were co-expressed with or without Myc-AKR1C3
in 293T cells. Flag-Siah2 was precipitated with anti-Flag M2 beads, and analyzed by western blotting with HA or Flag antibodies. The input protein was blotted with Myc or HA antibodies. **E.** Effect of AKR1C3 on Siah2 self-ubiquitination in vitro. For an in vitro ubiquitination reaction, GST-Siah2 was incubated with E1, E2, Ub in the presence of increasing amounts of His-AKR1C3. GST-Siah2 was then pulled down with glutathione beads and analyzed by western blotting with ubiquitin (upper panel) or GST (middle panel) antibodies. The input protein was blotted with His antibody. **F** and **G.** Effect of AKR1C3 on Siah2-mediated degradation of AR or NCO1. Flag-AR (F) or Flag-NCO1 (G) was co-expressed with indicated plasmids in 293T cells. Cell lysates were analyzed by western blotting with Flag, GFP or actin antibodies.

**Figure 6**

**A.** Representative images of sections of PCa TMA subjected to IHC for AKR1C3 (left panels) or Siah2 (right panels). Signals were visualized with DAB, and samples were counterstained with hematoxylin. Top panels: low AKR1C3 or Siah2 staining in benign prostate hyperplasia (BPH). Middle panels: low AKR1C3 or Siah2 staining in low-grade PCa. Bottom panels: high expression of AKR1C3 or Siah2 in a CRPC specimen. **B** and **C.** Quantification of AKR1C3 (B) or Siah2 (C) staining in PCa TMA. Staining was scored as 0 to 3 (0, no staining; 1, weak; 2, moderate; and 3, strong). Shown is the average staining score in BPH, indicated PCa types classified by Gleason (G) grade and CRPC. N= 33, 68, 51, 19, and 15 for BPH, G3, G4, G5, and CRPC, respectively. AKR1C3 or Siah2 staining was increased in G3 compared with BPH (p<0.0001 for AKR1C3, p<0.05 for Siah2), in G4 compared with G3 (p<0.05 for AKR1C3, p<5x10⁻⁶ for Siah2), in CRPC compared with G5 (p=0.06 for AKR1C3, p<0.01 for Siah2), but decreased in G5 compared with G4 (p<0.05 for AKR1C3, p<0.01 for Siah2). **D** and **E.** Kaplan-Meier curve analysis of PCa patients with high or low AKR1C3 staining (D) or with high or low Siah2 staining (E) for PSA recurrence after surgery or radiation therapy. For AKR1C3 or Siah2 staining, scores of 0 or 1 were classified as low expression, and scores of 2 or 3 as high. AKR1C3-high or Siah2-high groups had a quicker PSA recurrence (p<0.001 for AKR1C3-low vs AKR1C3-high, p=0.05 for Siah2-low vs Siah2-high).
Figure 1
Figure 2
Figure 3

A. Transcript levels

B. % of Input

C. Testosterone (ng/ml)

D. PSA transcript levels

E. PSA transcript levels

F. Absorption (MTT)

G. Testosterone (ng/ml)

H. Dihydrotestosterone (pg/ml)

I. Siah2 IP:

J. Absorption (MTT)

K. Colony number

L. Siah2 transcript levels

*Figure 3*
Figure 4
Figure 6

A. AKR1C3 and Siah2 staining images for BPH, PCa, and CRPC.

B. AKR1C3 staining score for BPH, G3, G4, G5, and CRPC.

C. Siah2 staining score for BPH, G3, G4, G5, and CRPC.

D. Kaplan-Meier plots for AKR1C3-low and AKR1C3-high groups.

E. Kaplan-Meier plots for Siah2-low and Siah2-high groups.
The steroidogenic enzyme AKR1C3 regulates stability of the ubiquitin ligase Siah2 in prostate cancer cells
Lingling Fan, Guihong Peng, Arif Hussain, Fazli Ladan, Emma Guns, Martin Gleave and Jianfei Qi

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