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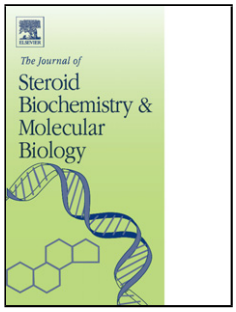

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Knockdown of AKR1C3 exposes a potential epigenetic susceptibility in prostate cancer cells.

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Short Title: AKR1C3 actions in prostate cancer.

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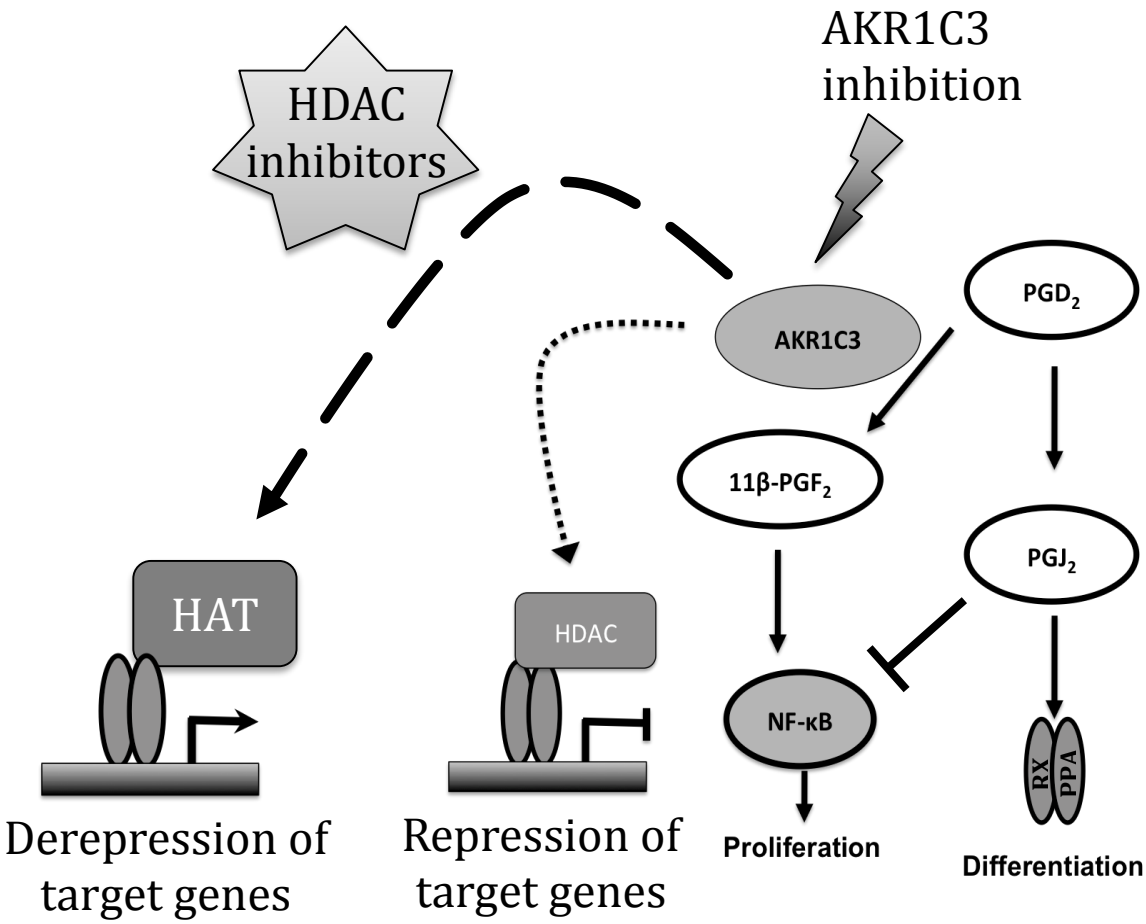
Graphical Abstract



Highlights

- AKR1C3 is overexpressed and linked to AR signaling in prostate cancer.
- Previous work suggests ligands for PPARs are an important target of AKR1C3.
- ShAKR1C3 had no effect on PPARs but enhanced sensitivity to HDAC inhibitors.
- AKR1C3 may regulate the epigenome and is a target alongside HDAC inhibition.

Abstract

Background. The aldo-keto reductase 1C3 (AKR1C3) has been heavily implicated in the propagation of prostate malignancy. AKR1C3 protein is elevated within prostate cancer tissue, it contributes to the formation of androgens and downstream stimulation of the androgen receptor (AR). Elevated expression of AKR1C3 is also reported in acute myeloid leukemia but the target nuclear receptors have been identified as members of the Peroxisome-Proliferator Activated Receptor (PPARs) subfamily. Thus, AKR1C3 cancer biology is likely to be tissue dependent and hormonally linked to the availability of ligands for both the steroidogenic and non-steroidogenic nuclear receptors.

Methods. In the current study we investigated the potential for AKR1C3 to regulate the availability of prostaglandin-derived ligands for PPARg mainly, Prostaglandin J_2 (PGJ_2). Using prostate cancer cell lines with stably reduced AKR1C3 levels we examined the impact of AKR1C3 upon proliferation mediated by PPAR ligands.

Results. These studies revealed knockdown of AKR1C3 had no effect upon the sensitivity of androgen receptor independent prostate cancer cells towards PPAR ligands. However, the reduction of levels of AKR1C3 was accompanied by a significantly reduced mRNA expression of a range of HDACs, transcriptional co-regulators, and increased sensitivity towards SAHA, a clinically approved histone deacetylase inhibitor.

Conclusions. These results suggest a hitherto unidentified link between AKR1C3 levels and the epigenetic status in prostate cancer cells. This raises an interesting possibility of a novel rational to target AKR1C3, the utilization of AKRIC3 selective inhibitors in combination with HDAC inhibition as part of novel epigenetic therapies in androgen deprivation therapy recurrent prostate cancer.

Keywords: Prostate cancer, 17B-Hydroxysteroid dehydrogenase, AKR1C3, PPAR, prostaglandins, Bezafibrate.

Introduction

Nuclear Receptors (NRs) regulate multiple gene targets controlling cell growth and differentiation in many self-renewing tissues including hematopoietic and epithelial cells. The ability of NRs to exert these gene regulatory effects is dictated by the supply of ligand availability and epigenetic context (reviewed in[1,2]).

Prostate cancer (CaP) represents an attractive disease with which to target NR signaling in either chemoprevention or chemotherapeutic strategies. Early stage disease is a potential target for chemotherapies that target NRs such as VDR, RARs/RXRs and PPARs[3-5]. Expression of these receptors is sustained in early stage CaP and they are well established as exerting a range of tumor repressive effects. For example, PPARg receptor activation induces anti-proliferative, pro-differentiating gene targets and has subsequently been the target of various clinical trials including a phase II clinical study with Troglitazone[6]. At late stage CaP targeting of the AR by androgen deprivation therapy (ADT) forms the current treatment mainstay for advanced prostate cancer. However, in ADT-R CaP (androgen deprivation therapy - recurrent prostate cancer), is predominantly lethal with limited alternative therapeutic strategies[7].

In late stage and ADT-R CaP aberrant co-repressor actions preclude NR activation, impairing anti-proliferative capacity and further epigenetic mechanisms contribute to this resistance[8] with similar events disrupting AR signaling[9,10] (reviewed in[11]). Specifically, PPARg actions are epigenetically disrupted and can be targeted selectively by using HDAC inhibitor co-treatments[12,13]. Elevated levels of the co-repressors NCOR1, and to a lesser extent NCOR2/SMRT, correlated with, and functionally drive, the selective insensitivity of PPARa/g receptors towards dietary derived and therapeutic ligands[13,14].

One approach to target NR signaling in CaP is to target the enzymes that regulate ligand availability. Aldoketoreductase 1C3 (AKR1C3) is a multifunctional enzyme and acts as a type 2 3-alpha-Hydroxysteroid dehydrogenase or 17-beta-HSD type 5. A key regulator of steroidal metabolism, it is implicated in cancer progression. For example, silencing of AKR1C3 has been shown to inhibit cervical cancer metastasis[15]. In CaP, AKR1C3 actions are implicated in the generation of androgens[16-19], AR activation and has been investigated as a potential biomarker for CaP progression[20],[21]. However, AKR1C3 is able to convert various different substrates and reflecting this, it has been implicated in the altered metabolism of chemotherapeutics propagating cancer cells resistance[22]. Perhaps reflecting this promiscuity over substrate choice, AKR1C3 is often overexpressed in prostate cancer tissues and prostate cancer cell lines[23]. Furthermore its expression is elevated in cell lines with either absent or low levels of AR[24] suggesting that

substrates may include those independent of androgen signaling; and 5α-Dihydrotestosterone may not be the only product of AKR1C3 [25,26].

Previous publications have considered the regulatory actions of AKR1C3 on alternative substrates, including the arachidonate-derived prostaglandins that act as *de novo* ligands for PPARg[27]. The expression level of AKR1C3 has been examined in ADT-R CaP tissue and cells lines, including PC-3 and DU 145 cells, and been shown to be elevated compared to less aggressive counterparts and directly proportional to the 11b-PGF_2 levels [28]. We and others have examined the ability of AKR1C3 to convert prostaglandin D_2 (PGD_2), into 9-alpha, 11beta-prostaglandin F_2 [28,29] (a ligand for the FP receptor, which is a driver of cell proliferation) preventing the alternative spontaneous and non-enzymatic generation of the potent PPARg ligand PGJ_2 [30,31]. Additionally, the PPARg mediated protective action of AKR1C3 have been investigated by exploiting 6-Medroxyprogesterone acetate (MPA) an inhibitor of AKR1C3 [32]. In leukemic systems a synergistic effect on cell death occurs with the combination of MPA with PPARg ligand bezafibrate [33]. Combinatorial treatments of MPA and PGD_2 have corroborated this in other cancer models to induce apoptosis and cell cycle arrest through PPARg driven activation pathways [34], and other studies have echoed this approach in CaP [35].

Therefore, the current study, examined the possibility that an AR-independent mechanism for AKR1C3 was operating in CaP cells and, in particular, we focused on a potential role in ADT-R CaP cells where AR signaling is either diminished or lost. By generating CaP cell lines stably expressing a short hairpin siRNA sequence to AKR1C3 we investigated responses to treatment with the PPARg ligand precursor and AKR1C3 substrate PGD_2.

Materials & Methods

Ligands. Suberoylanilide hydroxamic acid (SAHA) (Merck Inc, New Jersey, USA), GW9662 (Sigma-Aldrich) and Prostaglandin D_2 (Sigma-Aldrich) were stored in DMSO (Sigma-Aldrich) as 100mM stocks. Bezafibrate (PPARa/g), 6-medroxyprogesterone acetate (Sigma-Aldrich), and Indomethacin (Sigma-Aldrich) were stored as 10mM stocks in DMSO.

Cell Culture and shRNA Knockdown. ShRNA targeting AKR1C3 oligonucleotides containing the short hairpin sequence (Sigma-Aldrich) were annealed and inserted in a pcDNA3.1 vector (Invitrogen). Stably transfected cells were exposed to 100mg/ml Neomycin sulphate (Sigma-Aldrich) as a selection agent. Human prostate cell lines used RWPE-1, LNCaP, PC-3, DU 145 were purchased from American Type Cell Culture (ATCC) Manassas, Virginia USA. RWPE-1

cells were maintained within Keratinocyte serum free medium (K-SFM) (Invitrogen GIBCO) used in combination with the recommended supplements of bovine pituitary extract (0.05mg/ml) (BPE) and human recombinant epidermal growth factor (5ng/ml) (EGF). LNCaP, PC-3 and DU 145 cells were maintained in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (Invitrogen), 2mM L-Glutamine and containing 100 units/ml Penicillin and 100µg/ml Streptomycin. Cells were kept at 37ºC in 95% air and 5% CO_2. Cells were washed in sterile phosphate buffered saline (PBS) and split using Trypsin-EDTA (Sigma-Aldrich) and seeded into new flasks containing fresh media. All experiments were conducted using cells between passages 15 and 28.

Primary prostate tumor material. All tumors were collected under IRB approval at Roswell Park Cancer Institute (RPCI), specifically the Genitourinary Disease Site Research Network at RPCI, which assesses applications for non-human subject research under guidance of the Office of Research Subject Protection. All patients at RPCI give written consent to allow tumor material not needed for pathological grading to be considered for non-human subject research. Total mRNA from local tumors and adjacent non-neoplastic tissue from the same patient were extracted from snap frozen radical prostatectomy samples with subsequent frozen section analysis for quality control. The frozen section H&E was evaluated by a board certified pathologist for prostatic adenocarcinoma versus benign tissue. Segments of tissue corresponding to prostatic adenocarcinoma with equal to or greater than 70% neoplastic nuclei are submitted for RNA isolation. RNA processing was done in the Pathology Resource Network facilities with standard operating procedures as described previously [36].

Proliferation assays. Proliferation (ViaLight HS, LumiTech, Nottingham, U.K.) was measured as described previously [37] and optimized using different seeding densities to ensure exponential proliferation through the course of the experiment. Cancer cell lines ($2x10^3$ cells/well) and RWPE-1 ($4x10^3$ cells/well) were plated in 96-well, white-walled plates (Fisher Scientific Ltd., Loughborough, U.K.), dosed with agents to final volume of 100µl/well and incubated for 96hours, with re-dosing after 48hours. Cells were normalized to vehicle control treated wells performed in each separate assay plate. Each treatment was performed in technical triplicate and in biological triplicate experiments.

Q-RT-PCR. cDNA was prepared using random primers (Promega) and target genes relative expression quantitated using ABI 7500 - Applied Biosystems. Sequences for

AKR1C3 FORWARD GGGATCTCAACGAGACAAACG REVERSE AAAGGACTGGGTCCTCCAAGA PROBE TGGACCCGAACTCCCCCGGTG were designed

and validated. 18S VIC-labeled probe was used as an endogenous control (Applied Biosystems). Measurements were carried in triplicate, in triplicate wells for each condition and ddCt fold changes calculated.

Multi-target micro-fluidic Q-RT-PCRM. Measurement of targeted multiple gene transcripts was undertaken on custom-designed TaqMan Low Density Array (ABI 7900HT Fast Real-Time PCR System) as described previously [14] the full 95 gene list is available upon request. Briefly, the array included probes and primers for 18S and the gene targets in nine functional groups whose expression together reflects nuclear receptor signaling capacity. These were 1. Nuclear receptors (e.g. high and broad affinity such as *VDR* and *PPARs*); 2. Nuclear receptor co-factors (e.g. co-activators [p160 family, non-p160 members, members of the 'bridging' DRIP/TRAP complex], co-repressors [*NCOR1, NCOR2/SMRT, COPS2/TRIP15/Alien*]; 3. Histone modifiers (histone deacetylases [e.g. *HDACs hSIRT1*] and acetyltransferases [*P300, CBP, PCAF*], histone methyltransferases [*SUV39H1, SUV39H2*], demethylases [KDM1A/LSD1], Histone deaminases [e.g. *PAD14*]); 4. Metabolic enzymes (e.g. *CYP24* and *SULTA1*); 5. Cell death regulators (e.g. *CASP4* and *BAX*); 6. Transcription factors (e.g. *YY1* and *ID1*); 7. Cell surface transporters (e.g. ABC transporters such as *MRP3*); 8. Cell cycle regulators (e.g. *CCND1, GADD45a, CDKN1A, TP53*); 9. Signal transduction (e.g. *IGFBPs, MAPKs, CDH1*). The exact choices represented in classes 1-3 were guided by SAGE expression data from normal prostate tissue [38] and those in classes 4-9 included known direct target genes for nuclear receptors [39-46]. Total mRNA from cell-cycle sorted cells was quantified in triplicate samples measured in duplicate as described previously [14].

Fold changes were calculated for target gene expression and statistical analyses carried out using the TIGR MultiExperiment Viewer 4.0, MeV. A one sample t-test analysis based on permutation (Westfall Young stepdown [47] – MaxT correction) was used to identify genes significantly expressed. In this case vectors containing gene expression values were tested against the mean of 18S fold changes. One-Way ANOVA was used to identify genes that were differentially expressed for the shAKR1C3 PC-3 knock-down experiment compared to vector only PC-3 controls.

Western immunoblotting Total proteins were electrophoresed in a 12.5% gel, for 90 minutes at 120 volts. Proteins were then transferred onto PVDF (Polyvinylidene fluoride) membrane (45 minutes at 80 volts). The PVDF membranes were then left in 5% non-fat milk diluted in Tri-buffered saline containing 2% Tween (TBS-T) detergent for 45 minutes. Monoclonal anti-AKR1C3 antibody (Sigma-Aldrich) produced in mouse was diluted 1 in 10,000 into 5% non-fat

milk diluted in TBS-T. The PVDF membranes were left in the primary antibody mixture overnight in a cold room (5°C) to prevent evaporation. The PVDF membranes were washed in TBS-T for 15 minutes changing the TBS-T every 5 minutes. Secondary HRP-labeled anti-mouse immunoglobulin was diluted 1 in 10,000 into TBS-T and the membranes incubated in the mixture for 45 minutes. Chemoluminescent ECL western blotting detection kit (Amersham, GE Health Care) was added in accordance with manufacturer's protocol.

Statistical analysis. Data shown are mean ± SEM of at least three independent experiments with statistical significance defined as P <0.05 (*P < 0.05; **P < 0.01; ***P < 0.001) using unpaired Students T-test and were conducted with Prism (GraphPad, CA). Statistical analysis on real-time PCR data was performed on mean dct values.

Results

***AKR1C3* expression is elevated in prostate cancer cell lines and primary prostate cancer material. Figure 1A** illustrates Q-RT-PCR data for *AKR1C3* in CaP cell lines, normalized to RWPE-1 non-malignant prostate cells. Significant elevation of *AKR1C3* was demonstrated in PC-3 and LNCaP cancer cell lines (p <0.05); LNCaP (5.08 ± 1.0 SEM), PC-3 (7.54 ± 0.1 SEM). **Figure 1B** shows expression of *AKR1C3*, *PPARA* and *PPARG* in thirteen human primary tumors compared to matched normal tissue, derived from radical prostatectomy. There was a significant increase in AKR1C3 levels (2.69 ± 1.7 SEM). *PPARA* and *PPARG* levels were comparable between normal and tumor samples [48].

Neither AKR1C3 inhibition nor knockdown enhances PPAR anti-proliferative signaling. Previously, studies of acute myeloid leukemia have shown combinatorial anti-tumor activity when AKR1C3 inhibitors are combined with Bezafibrate (a dual PPARa/g ligand). Assuming prostate cell lines have comparable basal turnover of cholesterol-derived prostaglandin substrate the similar response would be observed. However, the use of AKR1C3 inhibitors either indomethacin or 6-medroxyprogesterone acetate [49] did not enhance the anti-proliferative effects of Bezafibrate against the non-malignant RWPE-1 cells or either CaP cell line (**Figure 2A**).

Stable transfection of short hairpin RNA targeted towards AKR1C3 in PC-3 and DU 145 resulted in significant reduction in both AKR1C3 mRNA (**Figure 2B)** and protein levels (**Figure 2C**). Cells were then treated with the PPARg agonist precursor PGD_2 to examine the biological impact of AKR1C3 reduction. **Figure 2D** demonstrates reduction of AKR1C3

levels had no significant affect on proliferative response to PGD_2 within PC-3 (Vector Only (VO) and shAKR1C3) and DU 145 (VO and shAKR1C3) cells. These data suggest that shAKR1C3 PC-3 and DU 145 cells have unchanged response to the PPARg ligand precursor PGD_2.

Cell proliferation in response to SAHA in shAKR1C3 transfected cells. Explanations for deficiency of AKR1C3 inhibitor impact may involve FP1 receptor expression and inherent variations in export/import of prostaglandins. However, we reasoned that the lack of a response to AKR1C3 inhibitors maybe due to epigenetic blockade of PPARg transcriptional actions. Therefore, the HDAC inhibitor acid SAHA was used to treat AKR1C3 knockdown cells in an attempt to overcome epigenetic resistance at PPARg targets. Down regulation of AKR1C3 in DU 145 cells resulted in significant sensitization to the anti-proliferative actions of SAHA. (**Figure 3 A and B).** A similar trend was observed in PC-3 ShAKR1C3 cells although this did not reach significance when compared SAHA treated PC-3 VO cells (**Figure 3 A and B).** The lesser difference in PC-3 cells may relate the to greater sensitivity of PC-3 VO to SAHA treatment alone when compared to DU 145 VO cells which displayed no antiproliferative response to SAHA. However, SAHA treatment did not sensitize shAKR1C3 cells to either the PPARg ligand precursor PGD_2 or the direct PPARg ligand GW9662. Interestingly, DU 145 cells revealed a significantly increased sensitivity to PGD_2 in shAKR1C3 cells. This reflects data on chemical inhibition of AKR1C3 by MPA leading to increased sensitivity to Bezafibrate (**Figure 2A**).

Multi-targeted micro-fluidic QT-RT-PCR of the NR network. The above findings in two prostate cancer cell line models suggest that the role of AKR1C3 in CaP is different from that in leukemia and is only weakly linked to the protection of PPARg. To reveal the impact of reduced AKR1C3 levels in this priming event we examined expression of the NR network using a previously established microfluidic PCR approach[8,14]. We selected PC-3 cells for these experiments as arguably it arguably is a more relevant model of ADT-RCaP than DU 145 cells, as it in vivo it will metastasize to the same sites as observed in human disease[50]. The most significantly deregulated genes in shAKR1C3 compared to VO PC-3 cells are shown in **Figure 4**. Interestingly, a common downregulation of a range of epigenetic regulators was observed, alongside AKR1C3 downregulation. These include HDACs, NR co-activators and NRs and PPARg coactivator (*PPARGC1A*). In addition the histone methyltransferase *SET7* and proto-oncogene *MYB* were significantly down-regulated in shAKR1C3. The NFkB complex component IKBkB was the only gene to be significantly

upregulated in the presence of shAKR1C3. Transcriptional activators forming the NFkB complex have been found to be important in prostate cancer progression and human prostate cancer prognosis [51-53]. This increase of NFKB in response to shAKR1C3 is of particular interest as it is a key driver of prostate cell proliferation and AKR1C3 presence influencing NFkB activity (through PGF_2 production) has also been suggested by others [54].

Discussion

The current study was undertaken to investigate the roles of AKR1C3 in CaP, as this enzyme is increasingly a focus in CaP research that includes its altered expression and function. The studies of others have focused on the role of this enzyme to alter ligand availability for steroidogenic androgen and estrogen receptors in the prostate. Additionally, studies and have considered the impact of PGD_2 on cell proliferation in cells that over-express AKR1C3 [55-59]. By contrast, our previous studies show elevated AKR1C3 deprives AML cells of J-series prostaglandins that can act as endogenous PPAR ligands. Given that PPARg ligands have been investigated for the treatment of various cancer types for example neuroblastomas [60], colorectal [61], breast and prostate cancers [62,63]. It was hypothesized that the role in CaP for AKR1C3 may be to silence PPAR signaling. Furthermore, it has been demonstrated that stable expression of AKR1C3 in hormone dependent MCF-7 breast cancer cell lines negatively impacts anti-proliferative actions of PGD_2 [27].

This study investigated AKR1C3 function by undertaking knock-down and chemical inhibition approaches in parallel to compare findings to previously published over-expression approaches [56,57]. In particular an overexpression investigation of AKR1C3 in PC-3 cells found overexpression promotes proliferation [35]. However, this study found PGD_2 exposure to overexpressing AKR1C2 and AKR1C3 PC-3 models significantly reduces proliferation compared to controls. However, in this study reduction of AKR1C3 in the presence of PGD_2 had no impact upon proliferation. This disparity reflects the unclear interplay between AKR1C2, AKR1C3 in prostaglandin metabolism.

PC-3 and DU 145 cells are derived from ADT-R CaP cells that are insensitive to AR signaling but retain the capacity for PPAR signaling. Therefore, knockdown of AKR1C3 in these models allows a clearer examination of the role of AKR1C3 in regulating PPARg. However, shAKR1C3 expression did not significantly alter responses to either endogenous (PGD_2) or synthetic PPARg (GW9662) ligands.

These data support the concept that either AKR1C3 is not a major regulator of PPARg in prostate cells or that in CaP the ability of AKR1C3 to exert such an action is overridden by epigenetic events[36,37,64]. Therefore SAHA was utilized in an attempt to restore PPARg function. Interestingly, the shAKR1C3 clones of DU 145 were significantly more sensitive to SAHA than controls; in PC-3 cells this was smaller and non-significant but trending in the same direction. This supports a hitherto unsuspected link between AKR1C3 actions and the epigenome. The anti-proliferative effects of combining AKR1C3 knockdown and SAHA treatment were not further enhanced in the presence of PGD_2, reinforcing the notion that these epigenetic actions of AKR1C3 in CaP cells are independent of PPARs. It is however, possible alternative routes of 15delta-PGJ_2 elimination in particular, conjugation and dismissal by glutathione could play a contributory role in the absence of PGD_2 response.

The fact that reduction of AKR1C3 levels lead to increased sensitivity towards SAHA suggested that AKR1C3 has a role in promoting CaP survival, proliferation and its removal whilst not directly affecting cell growth primes cells for sensitivity to other potential CaP therapeutics. This suggests overexpression of AKR1C3 has an impact upon the epigenetic state of the cell, and is independent of both PPAR and AR signaling, (given the diminished/absent AR state of these cells). The down regulation of a number of HDACs following AKR1C3 knockdown supports the hypothesis that AKR1C3 in some manner is able to regulate enzymes governing epigenetic gene regulation, explaining why the cells gain sensitivity to SAHA. Interestingly, the upregulation of IKBkB correlates with previous findings suggesting a link between AKR1C3 and NFkB signaling pathways [65,66]. More broadly to establish the association of AKR1C3 with tumor status The Cancer Genome Atlas (TCGA) data was examined to identify genes associated with AKR1C3 signalling, and in parallel the AR and PPARg signalling. The goal was to reveal commonly distorted components and to infer how AKR1C3 may be co-expressed with either of these pathways, and at what stage of prostate cancer. We mined RNA-Seq associated with AKR1C3 in two different cohorts of prostate cancer samples in TCGA, namely, the University of Michigan cohort of 94 advanced stage and invasive tumors [67] and the Stand Up to Cancer (SU2C) cohort of 118 metastatic castrate recurrent tumors [68]. From these analyses we generated heatmaps for each of the tumor cohort (Supplementary Figure 1). In each case AKR1C3 (red arrowhead) grouped by expression in a different cluster than the AR (Black arrowhead). In particular AKR1C3 expression clusters more closely with PPARs than the AR. These findings suggest that taking an unbiased approach to the networks in which AKR1C3 is

implicated supports a role of close association with PPARs rather than the AR and this is especially evident in aggressive and advanced disease that has failed anti-androgen treatment.

Furthermore in the SU2C cohort of 114 tumors, AKR1C3 is over-expressed and amplified in 4% of tumors. However network analyses[69] of AKR1C3 reveals a direct protein-protein interaction with ZHX1 which is a corepressor and known to recruit HDACs[70]. ZHX1[71] is a relatively unexplored protein. Large-scale protein-protein screens reveal that it interacts with proteins including histone deacetylase (HDAC1), the DNA methyltransferases (DNMT1 and DNMT3B)[72], and a number of transcription factors (NFYa and b) [73]. These findings generate confidence that a ZHX1 complex initiates chromatin condensation and leads to gene silencing. Together we believe strongly that our data generated by two different experimental approaches, and these parallel findings from others indicate a direct and functional link between AKR1C3 and the regulation of the epigenome.

The current study has revealed a novel role of AKR1C3 expression on the epigenetic status of prostate cancer cells that is independent of AR and PPARs signaling and highlights the potential for selective inhibitors of AKRIC3 in combination with SAHA or other HDAC inhibitors as prospective therapy in AR resistant disease (Figure 5).

Disclosure

All authors have nothing to disclose.

Author Contributions

CLD, SB and FK conducted the work. MJC and CLD wrote the manuscript. MJC and CMB conceived and designed the research.

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Figure captions

Figure 1. (A) AKR1C3 is elevated in most but not all prostate cancer cell lines and tissue samples. Total RNA from RWPE-1, LNCaP, PC-3 and DU 145 cells was extracted and reverse transcribed. Levels of *AKR1C3* mRNA were measured using Q-RT-PCR using ribosomal 18S as an endogenous control. Values were normalized relative to the non-malignant immortalized prostate RWPE-1 cell line shown as fold change in *AKR1C3*. Bars indicate mean values ± SEM of at least three independent biological measurements performed in triplicate PCR wells. **(B) AKR1C3 is elevated in most human prostate cancer tissue samples.** Thirteen samples of human prostate tumors were used and total mRNA extracted and subject to reverse transcription. The Q-RT-PCR was performed on the resulting cDNA using 18S as an endogenous control. The ddCt values for non-tumorigenic tissue samples were normalized and compared to human tumor samples. Statistical analysis was performed using t-test. *=p-value 0.05, **=p-value 0.01, ***=p-value 0.001.

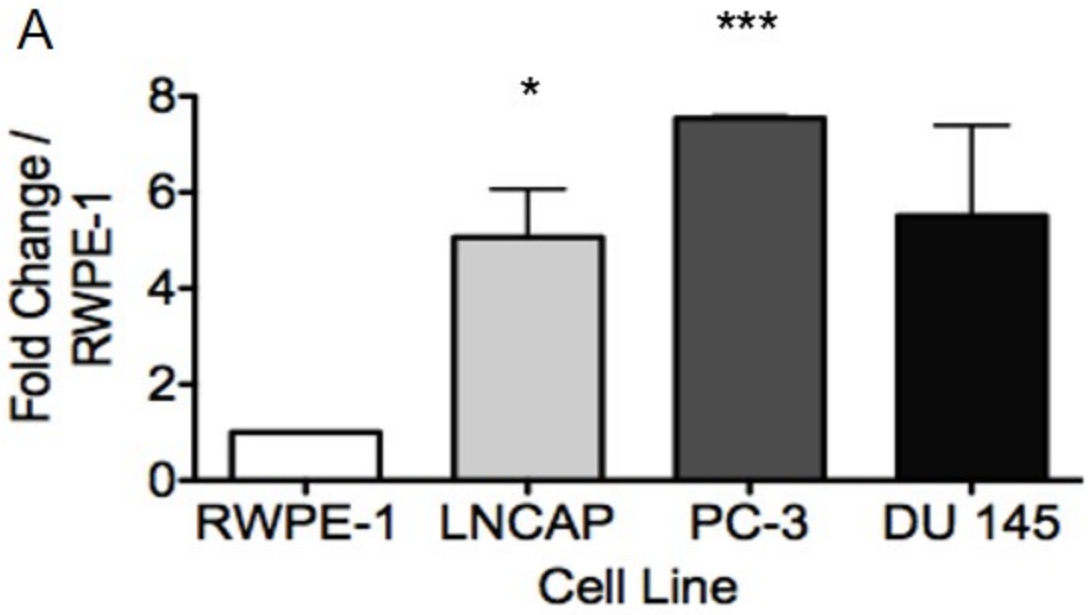
Figure 2. Reducing AKR1C3 levels is unable to restore anti-proliferative PGD_2 response. (A) RWPE-1, PC-3 and DU 145 cells were plated out at $2x10^3$ cells per well with bezafibrate (0.1μM) alone or in combination with indomethacin (20μM) or MPA (0.5μM). After incubation for 48 hours the cells were re-dosed again. Total treatment time was 96 hours. Bars indicate the mean of at least three independent experiments ±SEM. **(B)** PC-3 and DU 145 shAKR1C3 cells show significant reduction in *AKR1C3* mRNA and protein in comparison to vector only. Q-RT-PCR was performed on the resulting cDNA using 18S as an endogenous control. **(C)** DU 145 shows Western immunobloting analysis of shAKR1C3 transfected cells and vector only cells probed with AKR1C3 specific antibody and detected using chemoluminesence. Image shown is representative of three independent experiments.

(D) Cells were plated out at $2x10^3$ cells per well with PGD_2 added (0.62μM, 1.25μM, 2.5μM, or 5.0μM) and after incubation for 48 hours the cells were re-dosed with PGD_2. Total treatment time was 96 hours. Each data point represents the mean of four experiments each in triplicate wells ± SEM. Statistical analysis was performed using t-test *=p-value 0.05, **=p-value 0.01, ***=p-value 0.001.

Figure 3. The HDAC inhibitor SAHA shows no interaction with PPARg mediated signaling. (A) PC-3 and DU 145 cells were plated out at $2x10^3$ cells per well with PGD_2 (2.5μM) alone or in combination with SAHA (0.5μM). After incubation for 48 hours the cells were re-dosed again. Total treatment time was 96 hours. Bars indicate the mean of at least three independent experiments ±SEM. Statistical analysis was performed using t-test *=p-value 0.05, **=p-value 0.01, ***=p-value 0.001. **(B)** PC-3 and DU 145 cells were plated out at $2x10^3$ cells per well with GW9662 (1.0μM) alone or in combination with SAHA (0.5μM), after incubation for 48 hours the cells were re-dosed again. Total treatment time was 96 hours. Bars indicate the mean of at least three independent experiments ±SEM. Statistical analysis was performed using t-test *=p-value 0.05, **=p-value 0.01, ***=p-value 0.001.

Figure 4. AKR1C3 knockdown influences a network of genes involved in transcriptional regulation. Basal PC-3 vector only controls and shAKR1C3 PC-3 samples were used. Total mRNA was collected and added to a micro-fluidic reverse transcription Q-RT-PCR two step reaction. Primer sets on the card were selected for their nuclear receptor prostate specific context consisting of nine groups listed in the materials and methods section.

Figure 5. A model of AKR1C3 mediated epigenetic resistance in prostate cancer. (a) PGD_2 is responsible for the generation of PPARg ligand PGJ_2. (b) Increased AKR1C3 levels divert PGD_2 converting it to 11b-PGF_2 contributing to the activation of proliferative transcription factors such as the NFkB complex. (c) Observations in shAKR1C3 prostate cancer cells suggest AKR1C3 plays a role in sustained repression of anti-proliferative genes, this can be relieved with impairment of AKR1C3 activity combined with the treatment of HDAC inhibitor permitting access to the HATs responsible for permissive gene activation.


A
***
*
8
6
4
2
0
Fold Change / RWPE-1
RWPE-1
LNCAP
PC-3
DU 145
Cell Line

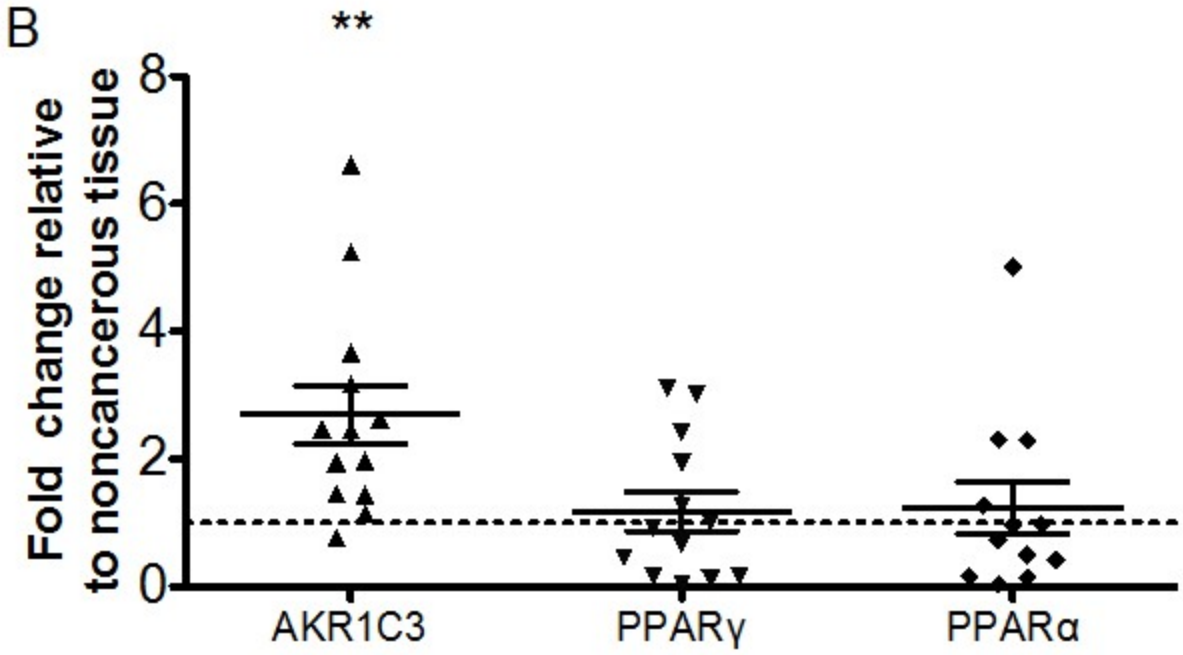

B
**
8
6
4
2
0
Fold change relative to noncancerous tissue
AKR1C3
PPARγ
PPARα

Figure 1

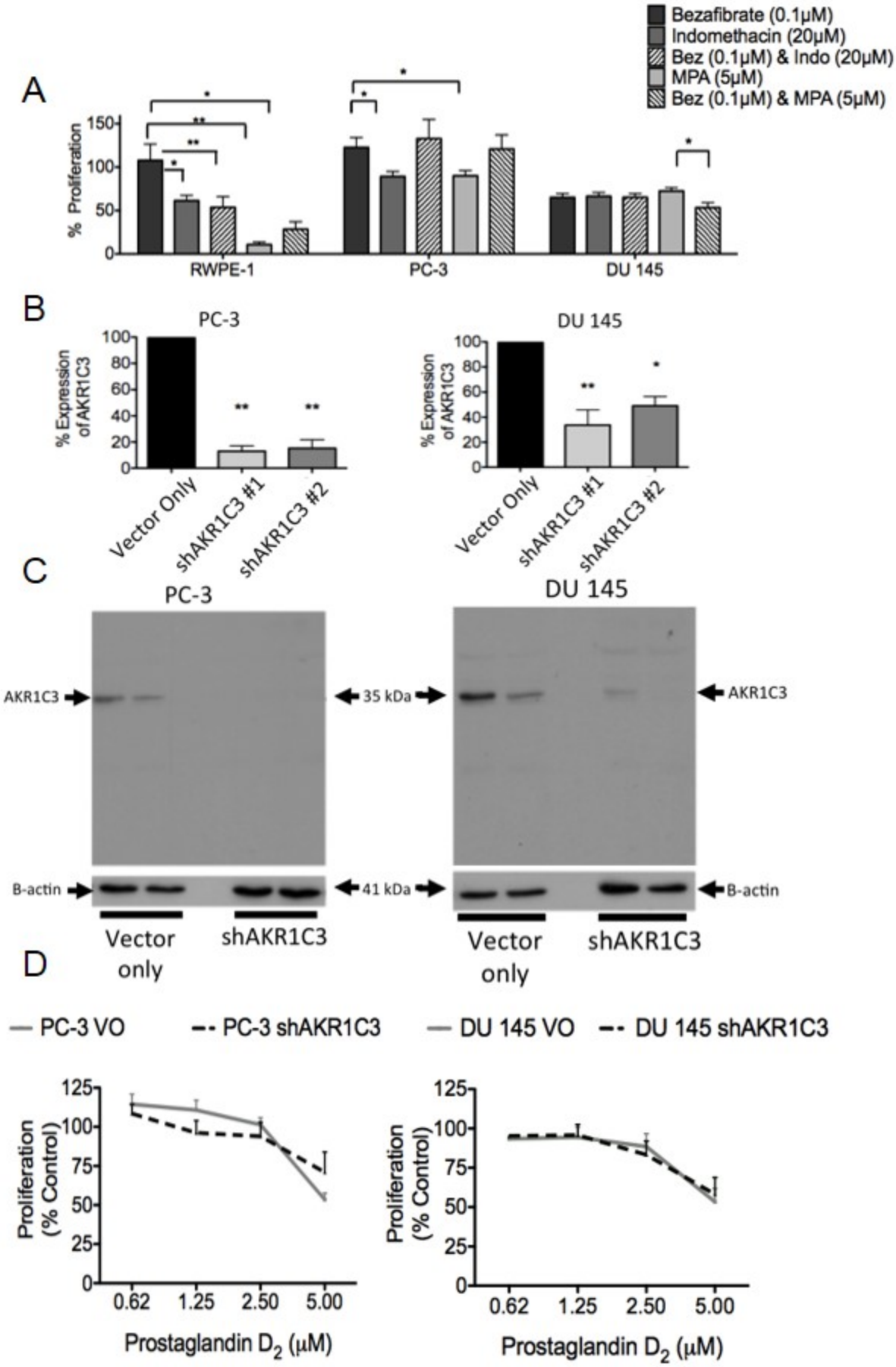

A
Bezafibrate (0.1μM)
Indomethacin (20μM)
Bez (0.1μM) & Indo (20μM)
MPA (5μM)
Bez (0.1μM) & MPA (5μM)
% Proliferation
RWPE-1
PC-3
DU 145
B
PC-3
DU 145
% Expression of AKR1C3
Vector Only
shAKR1C3 #1
shAKR1C3 #2
C
PC-3
DU 145
AKR1C3
35 kDa
B-actin
41 kDa
Vector only
shAKR1C3
D
PC-3 VO
PC-3 shAKR1C3
DU 145 VO
DU 145 shAKR1C3
Proliferation (% Control)
Prostaglandin D2 (μM)

Figure 2

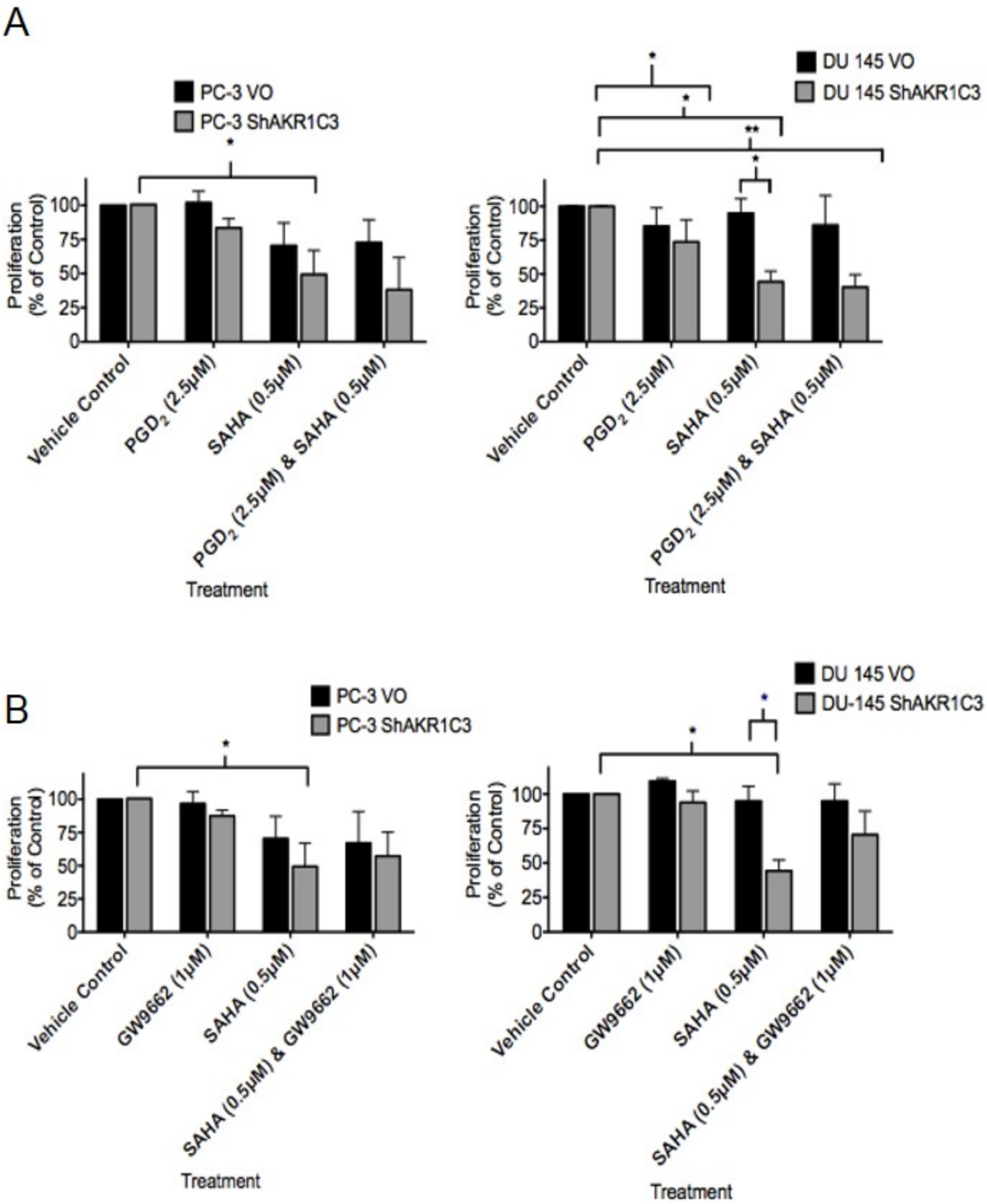

A
PC-3 VO
PC-3 ShAKR1C3
Proliferation (% of Control)
100
75
50
25
0
Vehicle Control
PGD2 (2.5μM)
SAHA (0.5μM)
PGD2 (2.5μM) & SAHA (0.5μM)
Treatment
DU 145 VO
DU 145 ShAKR1C3
B
PC-3 VO
PC-3 ShAKR1C3
GW9662 (1μM)
SAHA (0.5μM) & GW9662 (1μM)
DU 145 VO
DU-145 ShAKR1C3

Figure 3

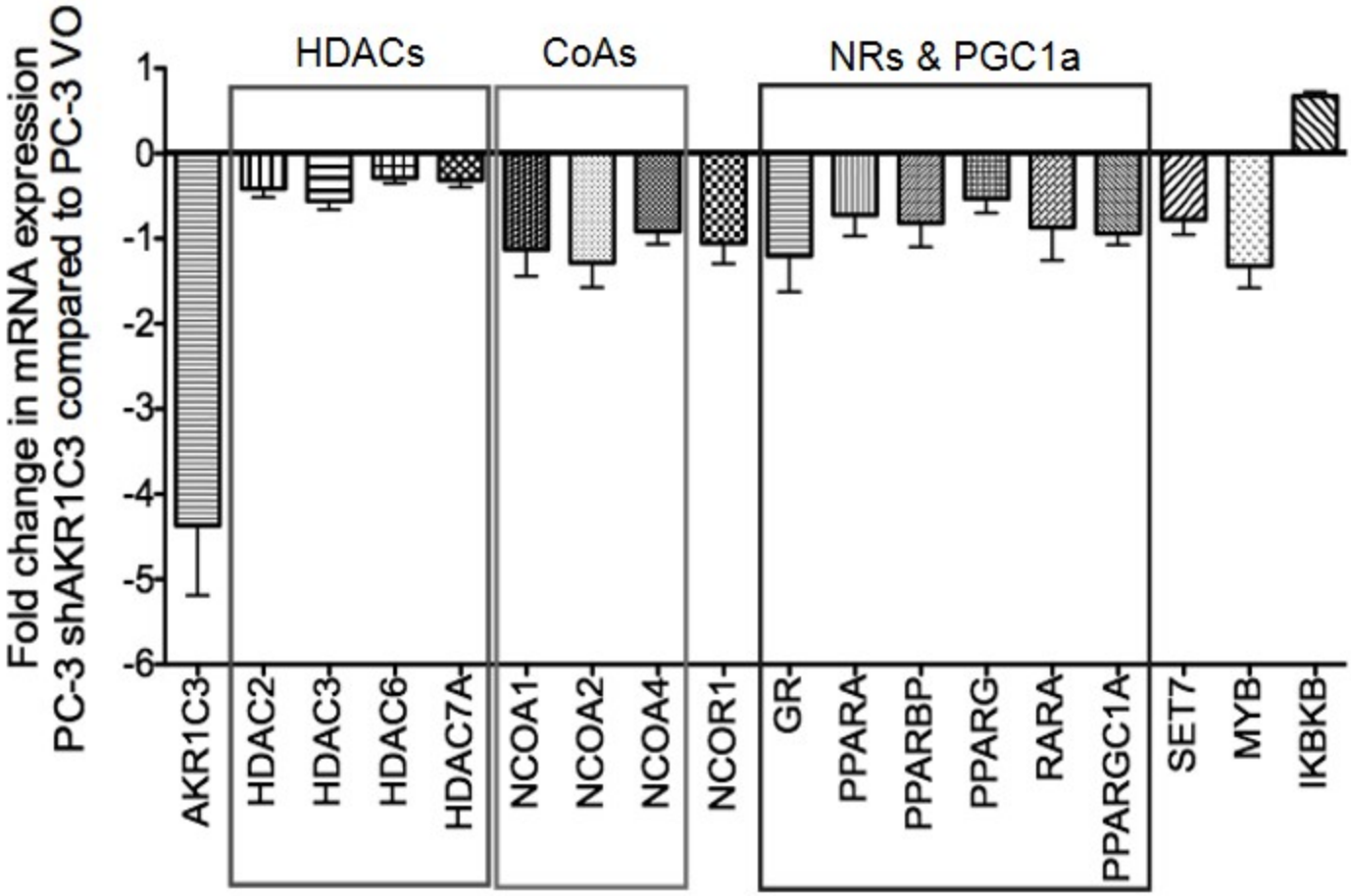

Fold change in mRNA expression
PC-3 shAKR1C3 compared to PC-3 VO
HDACs
CoAs
NRs & PGC1a
1
0
-1
-2
-3
-4
-5
-6
AKR1C3
HDAC2
HDAC3
HDAC6
HDAC7A
NCOA1
NCOA2
NCOA4
NCOR1
GR
PPARA
PPARBP
PPARG
RARA
PPARGC1A
SET7
MYB
IKBKB

Figure 4

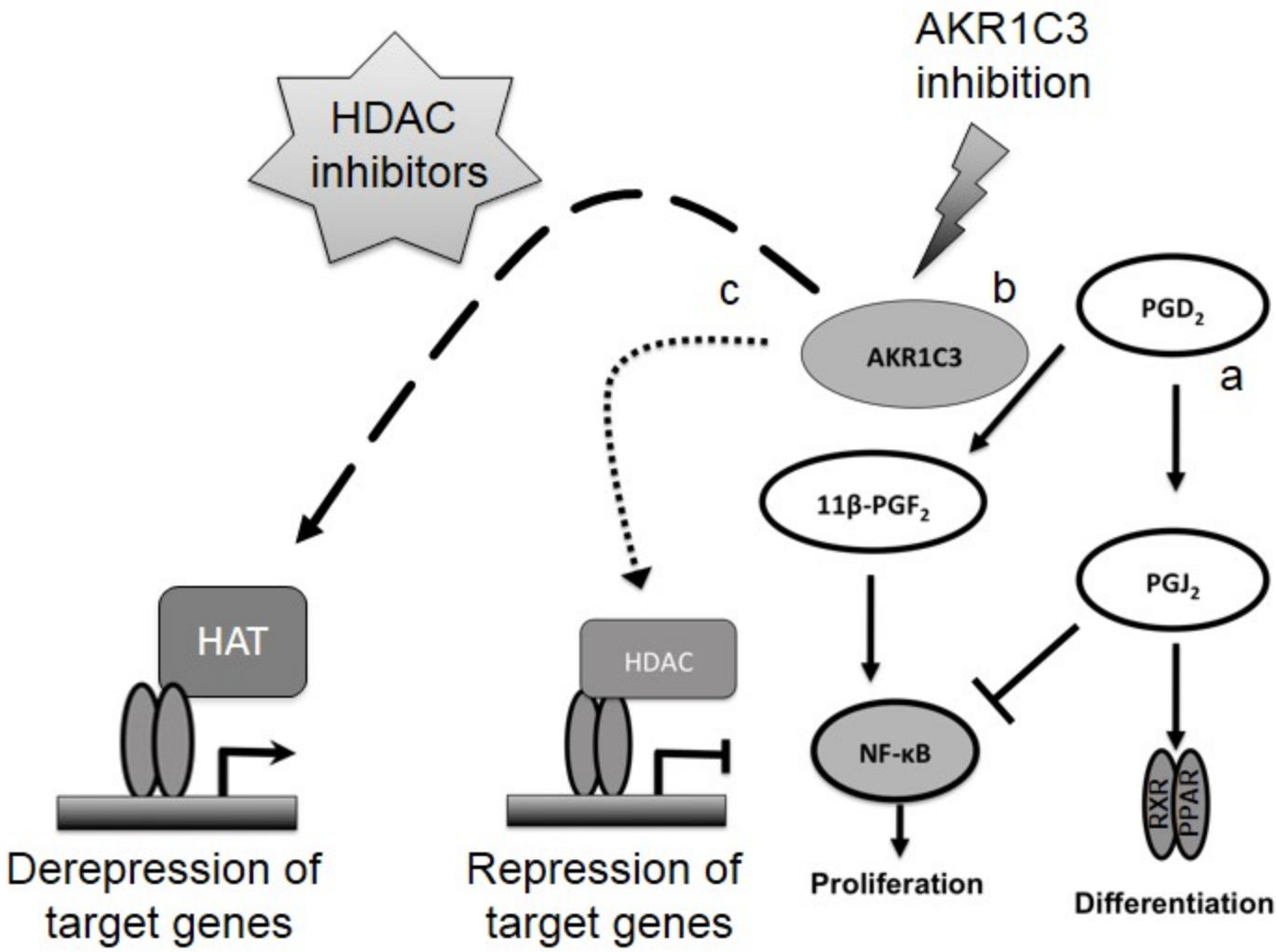

AKR1C3
inhibition
HDAC
inhibitors
c
b
AKR1C3
PGD$_2$
a
11β-PGF$_2$
PGJ$_2$
HAT
HDAC
NF-κB
RXR
PPAR
Derepression of
target genes
Repression of
target genes
Proliferation
Differentiation

Figure 5