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Original Article

Scaffold attachment factor B1 regulates androgen degradation pathways in prostate cancer

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Abstract: The nuclear matrix protein Scaffold Attachment Factor B1 (SAFB1, *SAFB*) can act in prostate cancer (PCa) as an androgen receptor (AR) co-repressor that functions through epigenetic silencing of AR targets, such as prostate specific antigen (PSA, *KLK3*). Genomic profiling of SAFB1-silenced PCa cells indicated that SAFB1 may play a role in modulating intracrine androgen levels through the regulation of UDP-glucuronosyltransferase (UGT) genes, which inactivate steroid hormones. Gene silencing of SAFB1 resulted in increased levels of free dihydrotestosterone (DHT), and increased resistance to the AR inhibitor enzalutamide. SAFB1 silencing suppressed expression of the UDP-glucuronosyltransferase family 2 member B15 gene (*UGT2B15*) and the closely related UGT2B17 gene, which encode proteins that irreversibly inactivate testosterone (T) and DHT. Analysis of human data indicated that genomic loss at the SAFB locus, or down-regulation of expression of the SAFB gene, is associated with aggressive PCa. These findings identify SAFB1 as an important regulator of androgen catabolism in PCa and suggest that loss or inactivation of this protein may promote AR activity by retention of active androgen in tumor cells.

Keywords: Androgen receptor (AR), SAFB1, UGT2B15, UGT2B17, dihydrotestosterone (DHT), prostate cancer, castration resistance

Introduction

The role of androgen and androgen receptor (AR) has been extensively studied in the context of prostate cancer (PCa). Standard medical treatment for aggressive PCa is androgen deprivation therapy (ADT), which is directed toward inhibition of the androgen axis. The classical model of disease progression is the re-emergence of AR activity even after (1) ADT and (2) resistance to next-generation AR and androgen synthesis inhibitors in castration resistant prostate cancer (CRPC). The AR is a key driver of progression to castration-resistance [1-7]. CRPC tumors frequently exhibit hyper-activated AR signaling, leading to the transcription of downstream target genes and tumor growth despite extremely low levels of circulating androgen in the patient. AR activation in CRPC arises from gene amplification (increased gene copy number, leading to increased mRNA expression), mutations within the AR ligand-binding domain (leading to ligand independence), and expression of AR splice variants that do not include a ligand-binding domain, but which are transcriptionally active [8].

We previously demonstrated that a chromatin-associated nuclear matrix protein, scaffold attachment factor B1 (SAFB1, SAFB), regulates AR activity and AR levels in in PCa [9]. SAFB1 was shown to be an AR co-regulator operating in concert with the serine-threonine kinase mammalian sterile STE20-like kinase 1 (MST1) and the enhancer of Zeste homolog 2/polycomb repressive complex2 (EZH2/PRC2) complex. Consistent with a trend toward decreased SAFB1 levels seen in human PCa specimens during disease progression, SAFB1 knockdown resulted in an aggressive phenotype and a tolerance for low androgen culture medium, con-

sistent with a role for SAFB1 as a tumor suppressor via inhibition of the AR axis [10]. Here we addressed the question of the physiological consequences of SAFB1 loss and report for the first time that SAFB1 plays a role in androgen catabolism, potentially impacting tumor-resident androgen levels during disease progression.

Materials and methods

Cell culture

The PCa cell lines used in this study were purchased from American Type Culture Collection (ATCC). LNCaP and 22Rv1 were routinely cultured in 75 cm^2 and 25 cm^2 tissue culture flasks (Greiner) at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines were cultured in Roswell Park Memorial Institute medium (RPMI) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Hyclone), and 1% penicillin/streptomycin (pen/strep) (Gibco).

Treatment of cells with dihydrotestosterone (DHT) or Enzalutatmide (MDV3100)

Full media was then replaced with 10% Charcoal stripped serum media (Gibco) in 1X RPMI-phenol red free media (Gibco) with 1% pen/strep for 24 hours. Aliquots of 10^{-6} M DHT was dissolved in absolute ethanol and added to the media at a range of final concentration of 10 nM for a further 24 hours. Experimental control conditions such as untreated and ethanol only (vehicle) were included in each experiment. MDV3100 (TRC Canada) was dissolved in DMSO and added to the media at a range of final concentration of 1-100 nM for a further 96 hours.

Stable knockdown of SAFB1

Stable gene knockdown was performed in both LNCaP and 22Rv1 cells using pre-validated shRNA from Mission Sigma-Aldrich. These predesigned shRNA targets the SAFB1 transcript (RefSeq: NM_002967). Gene knockdown was validated by qRT-PCR as well as western blot analysis of SAFB1 mRNA and protein expression.

shRNA hp1-sequence: sense strand-GCGCTACCATTCTGACTTTAA*;* antisense strand-TTAAAGTCAGAATGGTAGCGC; *shRNA hp2-sequence:* sense strand-GCAGATTGTGTCGAAGACGAT*;* antisense strand-ATCGTCTTCGACACAATCTGC.

Clonal selection was performed in a 96-well plate was based using an instruction provided by company (Corning).

Transient transfection

In a 6-well plate, 0.5 million cells were plated in full media for 24 hours. For androgen depletion, 10% charcoal stripped media was used to replace the full media and then a mix of 5 µg of vector construct and lipofectamine 3000 (Thermofisher Scientific) was added according to manufacturer's protocol.

Preparation of whole cell lysate

Whole cell lysates from LNCaP and 22Rv1 cells were prepared by lysis using 1X RIPA buffer SDS lysis buffer (Thermofisher Scientific). The cells lysates were incubated on ice for 5 minutes. The samples were then centrifuged at ~16,000×g for 15 minutes to pellet the cell debris.

Cytoplasmic and nuclear fractionation

Cytoplasmic and nuclear extracts were prepared as described [38].

Protein quantification

Protein concentrations of whole cell lysates in RIPA buffer or Cytoplasmic and Nuclear buffer were determined by a colorimetric assay following 1:10 dilution of protein in water. Protein quantification was performed according to the manufacturer's protocol and absorbance were read at 595 nm on SpectraMax 96 well microplate reader (Molecular Devices) on the SoftMax Pro software (Molecular Devices).

Western immunoblotting

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate proteins for Western immunoblot analysis. Polyacrylamide gels were purchased pre-cast 10 well- 4-20% Mini-PROTEAN III system (BioRad). Gels were run in 1X Tris/Glycine/SDS buffer (BioRad). The membranes were de-stained by washing in 1X PBST (0.1% Tween-20) prior to membrane blocking with 5% Nonfat-Dried Milk bovine (Sigma-Aldrich) at room temperature for 1 hour.

Primary antibody incubations were performed at an appropriate dilution in 5% Nonfat-Dried Milk bovine diluted in 1X PBS and 0.1% Tween-20 (PBST, Sigma-Aldrich). The ECL Western Blotting Substrate (Thermofisher Scientific) or SuperSignal West Femto (Thermofisher Scientific) detection system was used to allow the development of luminescence on antibody bound proteins. Membranes were exposed to X-ray films (Kodak) and autoradiographs were developed using Compact X4 film processor (Xograph). Primary Antibodies used for this study include SAFB1 (Sigma-Aldrich Mouse) 1:1000, AR (N-20, rabbit), UGT2B15 (Abcam, rabbit): 1:100, GAPDH-HRP conjugated (Cell Signaling) 1:1000.

Measurement of DHT depletion rate

The media from the cells were then collected at the time points 5 min, 2, 4, 6, 8 hours. The DHT from the media were separated by organic extraction (ethyl acetate) (90% + recovery rate). The non-metabolized radiolabeled DHT that was present within the organic layer were measured by scintillation detector as counts per minute (CPM). CPM was normalized to the 5 min time point and reported as % Free DHT.

Quantitative real time polymerase chain reaction

Total RNA was extracted from cultured cells using the RNAeasy isolation kit (Qiagen) following the manufacturer's protocol. RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (Thermofisher Scientific) and RNA samples stored at -80°C until ready for use. For reverse transcription, first strand cDNA was synthesized from total RNA by reverse transcription using the iScript Reverse Transcriptase kit (Bio-Rad). Data analysis was performed using the comparative Ct method normalized against β-actin expression. Experiments were performed in triplicate and statistical analysis was performed using student's t-test (Microsoft Excel). All effects at $P<0.05$ were reported as significant.

Primers

ABCA1-F-ACCCACCCTATGAACAACATGA; ABCA1-R-GAGTCGGGTAACGGAAACAGG; CYPA5-F-AATGTTTTGTCCTATCGTCAGGG; CYPA5-R-AGACCTTCGATTTGTGAAGACAG; DHRS8-F-CCTGCTTCTCCCGTTACTGAT; DHSR8-R-GATTTCGCCGGTGACTGATTT; UGT2B11-F-TCAGACATTCGAAAAGATAGC; UGT2B11-R-ATCTTTACAGAAGTTTCTAAATATGTCATAT; UGT2B15-F-CTTCTGAAAATTCTCGATAGATGGAT; UGT2B15-R-CATCTTTACAGAGCTTGTTACTGTAGTCAT; UGT2B17-F-TTTATGAAAATGTTCGATAGATGGAC; UGT2B17-R-CATCTTCACAGAGCTTTATATTATAGTCAG; UGT2B28-F-ACC GTT TGT GTA CAG TCT CT; UGT2B28-R-CAT TGT CTC AAA TAA TGT AGT G; SAFB1-F-TGGGGATGGGCAGGAGGATGTGGAG; SAFB1-R-ATGGTGAAGTCAGATGATGACGT; Beta-actin-F-CCCTGGCACCCAGCAC; Beta-actin-R-GCCGATCCACACGGAGTAC.

Statistical analysis

Data were represented as mean ± SEM wherever necessary. Student's t test (2-tailed) was used between the data pairs where it is appropriate. Either exact *p* value or a *p* value of 0.05 or less was considered significant and have been used. Significance of differential expression was calculated by Wilcoxon Rank-Sum test.

Results

A role for SAFB1 in androgen metabolism

Our previous findings indicate that SAFB1 is involved in the repression of cell and tumor growth, suppression of cell motility, resistance to anoikis, and growth in a hormone depleted environment [9]. In that study, the clinical significance of SAFB1 loss in PCa was also investigated, which revealed a significant decrease in SAFB1 protein staining intensity with disease progression (benign, locally confined and metastatic prostate cancer) [9]. Analysis of data from Grasso et al. [1] comparing copy number alterations (CNA) at the *SAFB1* and *AR* loci demonstrate a gain of *AR* and loss of *SAFB1* copy number in metastatic vs organ-primary PCa s (**Figure 1A**, left), consistent with our previous findings. Quantitative analysis of SAFB1 genomic loss showed a significant decrease of SAFB1 CNA ($P<0.0001$) in metastases (**Figure 1A**, right). These clinical data support an association between SAFB1 loss and disease progression.

To further characterize the relationship between SAFB1 and aggressive disease, we performed gene expression profiling in LNCaP cells to determine the functional consequences of

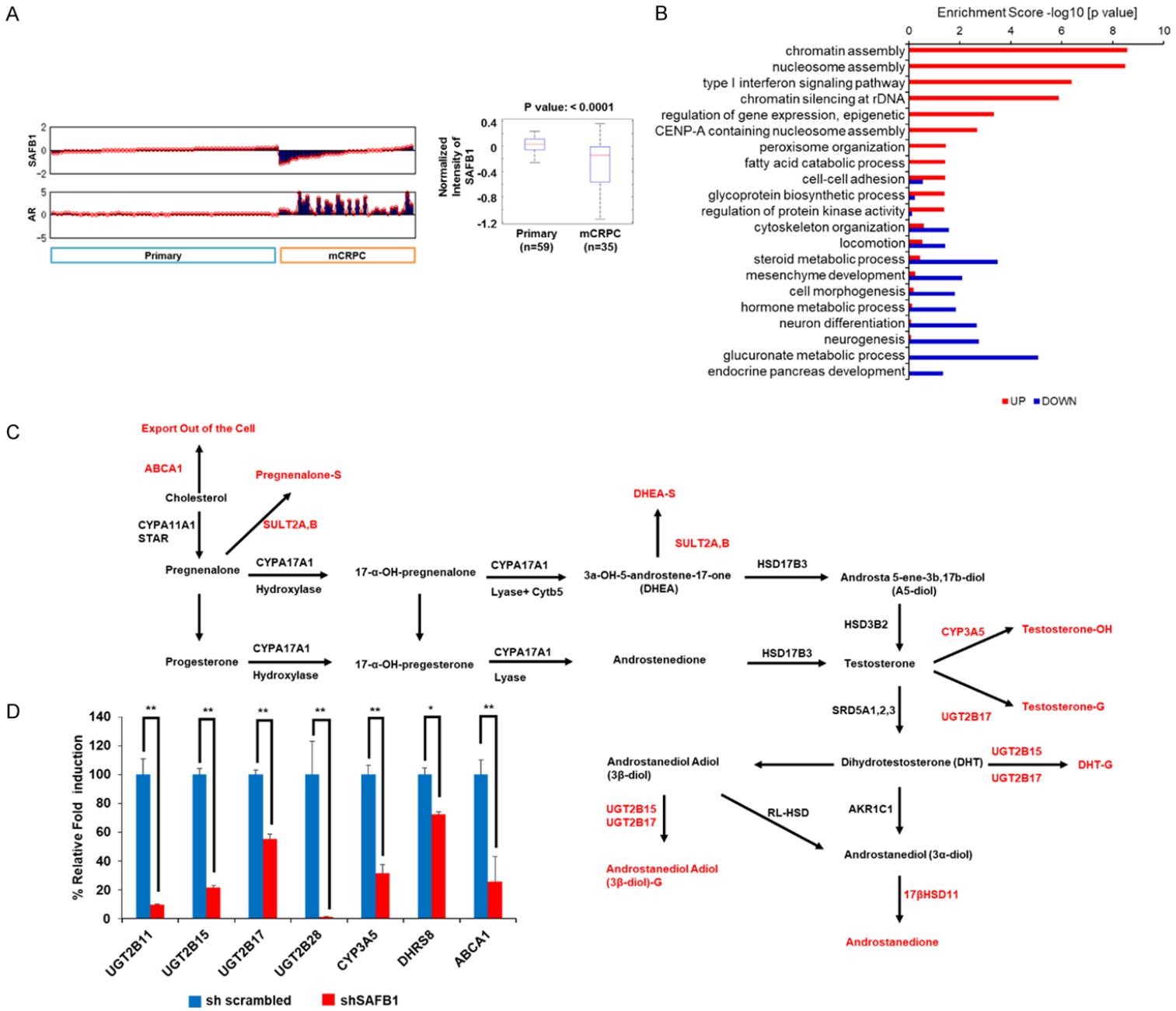
A
SAFB1
AR
Primary
mCRPC
P value: < 0.0001
Normalized Intensity of SAFB1
Primary (n=59)
mCRPC (n=35)
B
Enrichment Score -log10 [p value]
chromatin assembly
nucleosome assembly
type I interferon signaling pathway
chromatin silencing at rDNA
regulation of gene expression, epigenetic
CENP-A containing nucleosome assembly
peroxisome organization
fatty acid catabolic process
cell-cell adhesion
glycoprotein biosynthetic process
regulation of protein kinase activity
cytoskeleton organization
locomotion
steroid metabolic process
mesenchyme development
cell morphogenesis
hormone metabolic process
neuron differentiation
neurogenesis
glucuronate metabolic process
endocrine pancreas development
UP
DOWN
C
Export Out of the Cell
ABCA1
Cholesterol
Pregnenalone-S
CYPA11A1
STAR
SULT2A,B
Pregnenalone
CYPA17A1
Hydroxylase
17-α-OH-pregnenalone
CYPA17A1
Lyase+ Cytb5
3a-OH-5-androstene-17-one (DHEA)
DHEA-S
SULT2A,B
HSD17B3
Androsta 5-ene-3b,17b-diol (A5-diol)
HSD3B2
CYP3A5
Testosterone-OH
Progesterone
CYPA17A1
Hydroxylase
17-α-OH-pregesterone
CYPA17A1
Lyase
Androstenedione
HSD17B3
Testosterone
SRD5A1,2,3
UGT2B17
Testosterone-G
Androstanediol Adiol (3β-diol)
Dihydrotestosterone (DHT)
UGT2B15
UGT2B17
DHT-G
UGT2B15
UGT2B17
RL-HSD
AKR1C1
Androstanediol Adiol (3β-diol)-G
Androstanediol (3α-diol)
17βHSD11
Androstanedione
D
% Relative Fold induction
UGT2B11
UGT2B15
UGT2B17
UGT2B28
CYP3A5
DHRS8
ABCA1
sh scrambled
shSAFB1

Figure 1. Significant downregulation of SAFB1 in mCRPC may lead to a dysfunction in the steroid hormone metabolism pathway. (A) Copy number alteration in the Grasso et al. 2012 cohort [23] stratified by patients categorized as primary or mCRPC (LEFT) and the numerical normalized intensity of SAFB1 copy number alteration as a box-plot (RIGHT). (B) DNA microarray analysis of LNCaP cells stably knockdown for SAFB1 generated 922 DEGs, these genes were used for GO Enrichment analysis of biological processes that are induced (RED) and repressed (BLUE) by SAFB1 knockdown. (C) A diagram of genes involved in the classical steroid synthesis is mapped (BLACK) while DEGs selected from pathways that are repressed by SAFB1 silencing in LNCaP cells (RED) (D) quantitative Real-time PCR validation analysis of genes repressed by SAFB1 silencing. The data are normalized to beta-actin, which was not altered in the LNCaP cells silenced for SAFB1. in LNCaP cells, mean + SEM (n=3) *P<0.05, **P<0.005

SAFB1 loss. DNA microarray analysis of LNCaP cells stably silenced for SAFB1 revealed 922 differentially expressed genes (DEGs), of which 481 were upregulated and 441 were downregulated by SAFB1 depletion. The DEGs were selected based on the critera of a false discovery rate (FDR) <0.05 and a fold-change ≥ 1.5. We employed the DAVID (Database for Annotation, Visualization and Integrated Discovery) Gene Ontology (GO) tool [11, 12], using a *P*-value <0.05 to generate enrichement scores. In DAVID, the GO-BP FAT terms are used to report enrichment results [http://david.abcc.ncifcrf.gov]. The cellular processes represented by the DEGs were identified as enrichment scores calculated for cellular processes for the 481 upregulated genes (RED) and the 441 downregulated genes (BLUE) (**Figure 1B**). The biological processes most enriched for upregulated DEGs were: chromatin assembly, nucleosome assembly, chromatin silencing and epigenetic regulation. The upregulated biological processes are consistent with known roles of the scaffold attachment factor (SAF) family of proteins in the regulation of gene expression regulation and chromatin organization [13]. The biological pathways most enriched for the downregulated DEGs were glucuronidate metabolic processes, steroid hormone metabolic processes, and neuron differentiation. This finding is in agreement with our experimental findings [9] demonstrating that SAFB1 silencing conferred better cell growth in hormone-depleted media.

The DEGs identified as either up- or downregulated within each of the GO biological processes are listed in **Table 1**. Reviewing this list, we selected downregulated genes related to glucuronidate metabolism and steroid hormone metabolism for futher study of the role of SAFB1 in hormone regulation. Plotting the genes altered by SAFB1 depletion (RED) within the classic steroid hormone synthesis pathway (BLACK) showed that SAFB1 depletion suppressed the expression of genes encoding enzymes involved in the catabolism of active androgen metabolites. In contrast, SAFB1 depletion did not alter the expression of genes encoding enzymes that promote the synthesis of androgen metabolites (**Figure 1C**). To confirm this finding, quantitative real-time PCR was used to measure mRNA expression of the genes altered by SAFB1 depletion in LNCaP cells (RED). A series of genes relevant to steroid catabolism was confirmed to be downregulated by SAFB1 depletion (RED): UDP-glucuronosyltransferase 2B11 (UGT2B11), UGT2B15, UGT2B17, UGT2B28, Cytochrome P450 3A5 (CYP3A5), ATP Binding Cassette Subfamily A Member (ABCA1) (P<0.005) and Type 11 hydroxysteroid (17-β) dehydrogenase (HSD17B11/DHRS8) (P<0.05) (**Figure 1D**). Sulfotransferase 2B1 (SULT2B1) did not show any significant difference between SAFB1-depleted and control cells (not shown). UGT2B11, UGT2B15, UGT2B17, UGT2B28 are enzymes that covalently modify their substrates with glucuronic acid, thereby inactivating the targets and making them more hydrophilic and suitable for excretion [14]. UGT2B17 [14, 15] can conjugate testosterone (T), dihydrotestosterone (DHT), androstanediol, and androsterone, while UGT2B15 [15, 16] can conjugate T, DHT, and androstanediol. These findings revealed that suppression of SAFB1 expression results in down-regulation of genes involved in androgen degradation.

SAFB1 silencing suppresses androgen-inactivating genes UGT2B15 and UGT2B17

In order to confirm the effects on UGT2B15 and UGT2B17, SAFB1 was silenced using lentiviral-mediated RNA interference with two independent small hairpin RNAs (shRNA) in AR-postive LNCaP (hormone-dependent) and 22Rv1 (castration-resistant) cell lines. A heat-map of the 50 most up-regulated DEGs and 50 most down-regulated DEGs from the SAFB1 depletion in LNCaP cells is shown in **Figure 2A**. A notable finding is that all of the members of the UGT2B family of enzymes expressed in the prostate,

Table 1. List of the up and downregulated DEGs genes altered by SAFB1 silencing in LNCaP for each GO-FAT biological process

Terms	Up-regulated DEGs	Down-regulated DEGs
chromatin assembly	CENPN, HIST1H2BC, HIST1H2BD, HIST1H1C, HIST1H2BE, HIST1H2BF, HIST2H4A, HIST2H3C, HIST2H4B, TSPYL2, CDKN2A, HIST1H2BK, HIST2H2BE, HIST1H4B, HIST1H2BJ, HIST1H4E, HIST1H3D, HIST1H3F, HIST1H3H, HIST1H4H	NA
nucleosome assembly	CENPN, HIST1H2BC, HIST1H2BD, HIST1H1C, HIST1H2BE, HIST1H2BF, HIST2H4A, HIST2H3C, HIST2H4B, TSPYL2, HIST1H2BK, HIST2H2BE, HIST1H4B, HIST1H2BJ, HIST1H4E, HIST1H3D, HIST1H3F, HIST1H3H, HIST1H4H	NA
type I interferon signaling pathway	IFIT3, IRF9, HLA-H, IFIT1, IRF6, IFITM2, IFITM3, HLA-B, IFI35, GBP2, PSMB8, IFI6	NA
chromatin silencing at rDNA	HIST1H4B, HIST1H4E, HIST1H3D, HIST1H3F, HIST2H4A, HIST2H3C, HIST1H3H, HIST2H4B, HIST1H4H	NA
regulation of gene expression, epigenetic	HIST1H2AC, H2AFJ, HIST2H4A, HIST2H3C, HIST2H4B, MOV10, UHRF2, HIST1H4B, HIST1H2AI, HIST1H4E, ZC3H12A, HIST1H3D, TWISTNB, HIST1H3F, HIST1H3H, HIST1H4H	PHF19, H2AFY, FXR1
CENP-A containing nucleosome assembly	CENPN, HIST1H4B, HIST1H4E, HIST2H4A, HIST2H4B, HIST1H4H	NA
peroxisome organization	PEX11A, PEX11B, TMEM135, PEX10	NA
fatty acid catabolic process	MUT, AMACR, HSD17B4, PCCA, TWIST1, AUH	NA
cell-cell adhesion	RTN4, CAST, MPZL2, GCNT2, CADM1, PDLIM5, HLA-DRB3, CALD1, FKBP1A, SDC4, CHD7, CDKN2A, SH3GLB1, TTYH1, SMAGP, MAP2K5, ADAM9, COBLL1, TNFSF4, MYO1B, SWAP70, IL1RN, EFNB2, DUSP22, ELMO2, NCAM2, IGSF5, BTN3A1, ATP2C1, NLGN4X, HIST1H3D, SEMA4D, HIST1H3F, HIST1H3H, LCP1	ASS1, MAFB, ELF4, PCDH20, F2RL1, FSCN1, SOX4, CELSR3, CITED2, RPS26, PAK2, SERPINE2, NLGN4Y, CDH18, FAT1, RAC1, SDCBP, BCL6, NDRG1, HSPD1, LAMB1, CDH26, IGSF9, IL1RAPL1
glycoprotein biosynthetic process	MGAT4B, OSTC, GCNT2, B3GALT4, DPY19L4, PARP10, VEGFB, PGM3, GALNT10, DSEL, BMPR1B, THBS1, GALNT11, B4GALT5	ST6GAL1, POMT1, CHST3, MAN1C1, ART1, MUC4, PHLDA1
regulation of protein kinase activity	IBTK, TESC, FGR, SOCS2, SOCS1, TAOK3, DUSP22, TPD52L1, PKIB, SDC4, CAMK2N1, MAGED1, SPRY1, TSPYL2, CDKN1B, CDKN2A, AIDA, ADRA2A, EFNA5, SPRED1, THBS1, ADAM9, MVP, MAP2K5	GCG, ADCY1, MAP4K5, PAK2, ZAK, LRRTM4, PSMD10, MMD, H2AFY, UBE2C, PKIA, MAP2K6
cytoskeleton organization	ABLIM1, COBL, ODAM, GPM6B, SDC4, SPRY1, ARPC3, ADRA2A, SKA2, NEFL, RASA1, COBLL1, MYO1B, SWAP70, ZMYM6, DPYSL3, MID1IP1, ELMO2, ATF5, MAST4, KRT19, CDKN1B, ATP2C1, CC2D2A, TMSB4X, EFNA5, MAP7, PDCD6IP, LCP1	KRT6C, PRPH, PALM, ZAK, F2RL1, RHOU, DSTN, CORO2A, FAT1, RAC1, TUBA3D, BCL6, TUBA3E, TUBA1A, TUBB3, TMEFF2, SGK1, CAP2, CRYAB, FSCN1, MAP1B, ARHGAP28, EPHA3, SEMA6A, SVIL, FHOD3, SDCBP, WDR1, HAUS8, ANTXR1
Terms	Up-regulated DEGs	Down-regulated DEGs
locomotion	RTN4, GCNT2, FGR, JAG1, SDC4, SBDS, ADRA2A, RRAS, ZC3H12A, NOS3, THBS1, NEFL, MAP2K5, TWIST1, ADAM9, PLP2, SWAP70, ARID5B, EFNB2, PTPRR, ADIPOR1, ARTN, DPYSL3, SYNJ2BP, ANXA5, ELMO2, VEGFB, PRKD1, PARP9, SRGAP3, EFNA5, TMSB4X, SEMA4D, LAMC1, BMPR1B, LCP1, TP53INP1	ATP1B1, NRTN, NDN, OPRK1, STAT5A, F2RL1, ONECUT2, EPHB4, CITED2, GLIPR2, SERPINE2, HOXA5, FAT1, RAC1, ROBO3, LAMB1, ANGPT2, TUBB3, TMEFF2, ST6GAL1, SGK1, FSCN1, CELSR3, DPYSL4, FAM60A, EPHA3, SMO, LAMA1, SEMA6A, LAMA3, ID2, BTG1, RRAS2, SDCBP, FOXC2, SERPIND1, MERTK
steroid metabolic process	APOL2, TNFSF4, HMGCS2, AMACR, EPHX2, ERLIN1, HSD17B4, ABCG1	CYP3A5, HMGCS1, FDPS, SULT2B1, ATP1A1, ABCA1, FDFT1, UGT2B17, SQLE, INSIG1, UGT2B15, WWOX, UGT2B7, BMP6, FABP6
mesenchyme development	RTN4, GCNT2, ADIPOR1, JAG1, SEMA4D, TWIST1	GLIPR2, SMO, AMH, SEMA6A, NRTN, HOXA5, SDCBP, FOXC2, TEAD2, RBPJ, CITED2
cell morphogenesis	ABLIM1, RTN4, COBL, GCNT2, FGR, PDLIM5, OCRL, RIMS1, MBP, COL4A3BP, GALNT11, NEFL, RASA1, TWIST1, EFNB2, NTNG1, ARTN, ADIPOR1, ATMIN, SCFD1, CC2D2A, CFDP1, EFNA5, SEMA4D, LAMC1, BMPR1B	PALM, ADCY1, SYT4, NDN, ONECUT2, RHOU, GLIPR2, RAC1, BCL6, ROBO3, LAMB1, SPATA6, IL1RAPL1, TUBB3, ST6GAL1, SGK1, CAP2, MAP1B, DPYSL4, CELSR3, S100A13, CAPRIN2, EPHA3, SMO, SEMA6A, SLITRK3, ID2, ST14, LRRN1, SDCBP, ANTXR1, RBPJ, MERTK
hormone metabolic process	RDH11, MME, HSD17B4, DDO	TTR, STC2, SAFB, UGT2B11, ATP1A1, DIO1, PCSK6, UGT2B7, BMP6

neuron differentiation	RTN4, COBL, CPEB3, PDLIM5, EFNB2, PTPRR, NTNG1, ARTN, DPYSL3, JAG1, SDC4, RIMS1, MBP, PRKD1, ATF5, NCAM2, TULP3, BTG2, NLGN4X, EFNA5, SEMA4D, BMPR1B, CHD5, NEFL	GPRIN1, IRX3, PALM, NRTN, ADCY1, SYT4, NDN, ONECUT2, MMD, SOX4, SERPINE2, PAK2, RAC1, BLOC1S1, BCL6, ROBO3, LAMB1, IL1RAPL1, TUBB3, PHLDA1, DFNA5, SGK1, MAP1B, CHST3, DPYSL4, CELSR3, CAPRIN2, EPHA3, SMO, SEMA6A, SLITRK3, ID2, LRRN1, RIT2, RBPJ, IGSF9, BMP6
neurogenesis	RTN4, COBL, CPEB3, PDLIM5, JAG1, SDC4, RIMS1, MBP, CHD7, NEFL, CHD5, TWIST1, EFNB2, MPP5, NTNG1, PTPRR, ARTN, DPYSL3, PRKD1, ATF5, NCAM2, TULP3, BTG2, NLGN4X, EFNA5, SEMA4D, BMPR1B, BIN1	GPRIN1, IRX3, PALM, NRTN, ADCY1, NDN, SYT4, ONECUT2, MMD, SOX4, SERPINE2, PAK2, BCHE, SMARCD3, RAC1, BLOC1S1, BCL6, NDRG1, ROBO3, LAMB1, IL1RAPL1, TUBB3, PHLDA1, DFNA5, SGK1, MAP1B, CHST3, CELSR3, DPYSL4, CAPRIN2, EPHA3, SMO, SEMA6A, SLITRK3, ZNF217, ID2, LRRN1, WDR1, RIT2, RBPJ, IGSF9, BMP6
glucuronate metabolic process	NA	UGT2B17, UGT2B11, UGT2B10, UGT2B15, UGT2B28, UGT2B7, XYLB
endocrine pancreas development	NA	SMO, ONECUT2, SOX4, BMP6

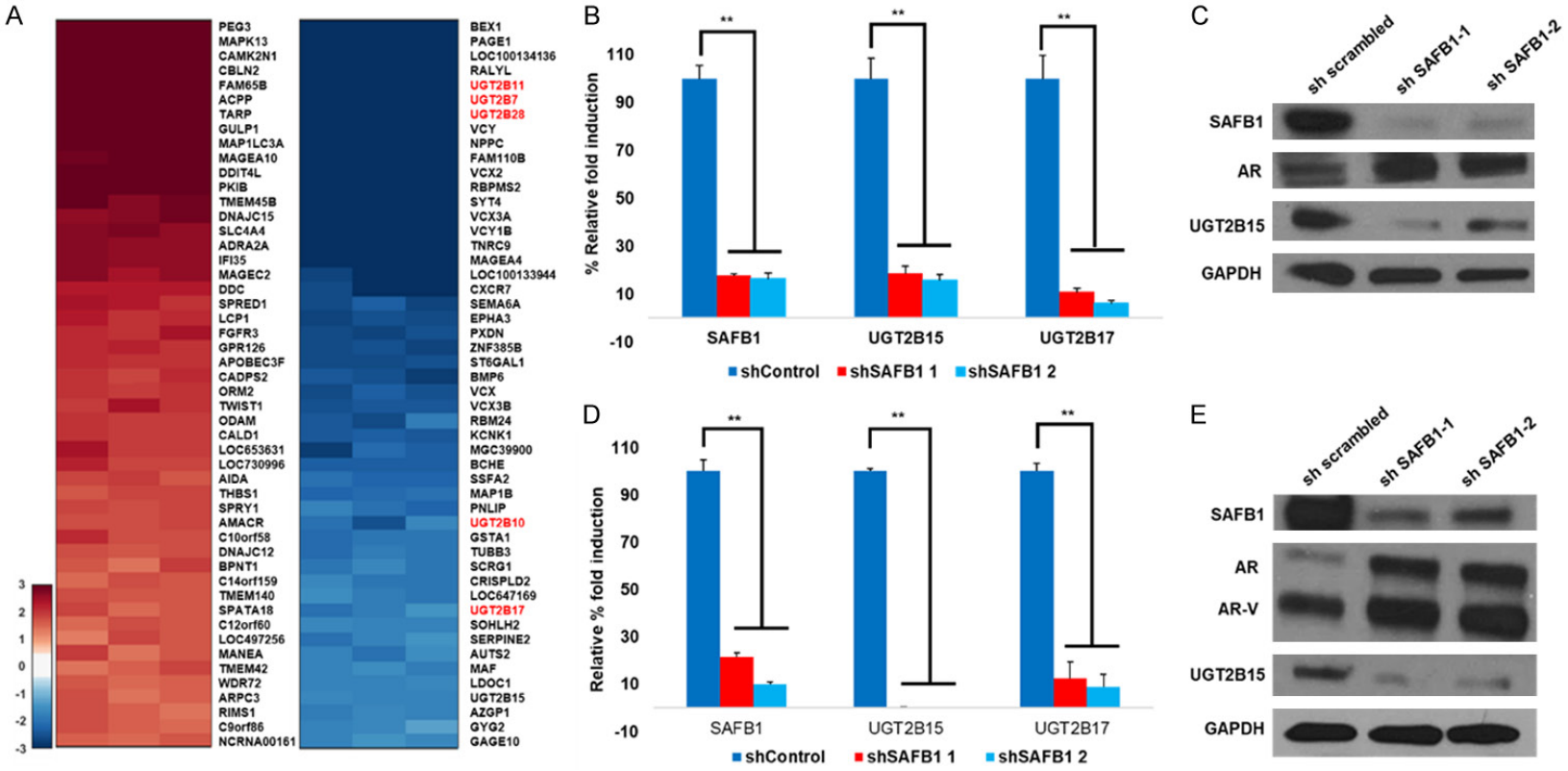

Figure 2. SAFB1 silencing downregulates a family of UGT2B enzymes involved in catabolism of steroids. (A) Heatmap (n=3) depicts differential expression patterns of genes induced (RED) and repressed (BLUE) by SAFB1 silencing, scale of intensity shown to the left. In LNCaP and 22Rv1 using two independent shRNA specific

to SAFB1 were used and analyzed by quantitative real-time PCR. SAFB1, UGT2B15 and UGT2B17 mRNA expression levels were significantly decreased for both (B) LNCaP and (C) 22Rv1 cells. Western blot analysis also shows that SAFB1, UGT2B15 and AR protein level reduction in both (D) LNCaP and in (E) 22Rv1 there is also a reduction in AR-V. For (A), microarray cut-off log fold change ≥ 1.5 was classified as DEGs. For (B and C) mean ± SEM (n=3). *P<0.05, **P<0.005.

with the exception of UGT2B4, were among the 50 most downregulated genes when SAFB1 was knocked down. Among these family members, UGT2B15 and UGT2B17 are well described as mediators of the irreversible glucuronidation of active androgen substrates (primarily T and DHT) [17-19]. Covalent modification of T and DHT through the addition of glucuronic acid to the steroid substrate allows the product to be released into the circulation and readily excreted through bile or urine, thereby preventing ligand-dependent activation of the AR [14]. Genomic alterations of these enzymes has been shown to be associated with variations in circulating steroid levels in PCa patients [20, 21]. Down-regulation of these genes would suppress androgen degradation pathways.

Downregulation of UGT2B15 and UGT2B17 mRNA expression (P<0.005) and visible reduction of UGT2B15 protein levels were seen in both cell lines (**Figure 2B** and **2D**). Consistent with previous findings, AR mRNA and protein levels increased when SAFB1 was silenced (**Figure 2C** and **2E**). Interestingly, SAFB1 silencing in 22Rv1 cells also increased levels of the 75 kDa AR variant AR-V7, which is associated with CRPC.

SAFB1 loss in aggressive prostate cancer and reduced clearance of androgen

Upregulation of the AR seen with SAFB1 silencing (**Figure 2**) suggests the potential for an association of SAFB1 loss/down-regulation with poor prognosis and disease progression. We have reported an association between SAFB1 loss and aggressive PCa. Next we sought to further charaterize this phenotype by exploring SAFB1 loss and its effect on patient outcome, treatment resistance, and whether the accumulation of the metabolite DHT was consistent with the data from SAFB1 silencing. To investigate patient outcome, we employed disease-free survival analysis of The Cancer Genome Atlas (TCGA) data [22]. Separation of patients with SAFB1 loss compared to wild type were analyzed for disease-free survival. Analysis of The Cancer Genome Atlas (TCGA) data [22] showed a significant decrease in disease-free survival for patients with genomic loss at the *SAFB* locus (p=0.0186) (**Figure 3A**). Analysis of the SU2C/PCF dream team cohort of metastatic PCa [23] showed that patients treated either with hormone suppression or taxanes showed a significant decrease in SAFB1 mRNA expression in comparison to untreated patients (**Figure 3B**). These findings suggest the possibility that SAFB1 silencing promotes treatment resistance. Consistent with this, in the presence of enzalutamide we observed increased growth of LNCaP cells silenced for SAFB1 compared with control cells (**Figure 3C**), which exhibited a cytostatic response as described [24].

With SAFB1 silencing, UGT2B15/UGT2B17 mRNA and UGT2B15 protein levels were reduced, suggesting that androgen degradation was attenuated. Microarray expression experiments also predict a decrease in the degradation of AR activating ligands in the context of SAFB1 down-regulation. Consequently, we measured the impact of SAFB1 depletion on levels of DHT through quantification of DHT by high-pressure liquid chromatography (HPLC). Cells depleted of SAFB1 showed a significantly slower rate of loss of radiolabeled DHT over time in comparison to control cells (**Figure 3D**), suggesting a reduction in the capacity to catabolize androgen. These results are consistent with observations that these cells grow even in low androgen environments [9] and are enzalutamide resistant.

Potential AR co-factors in regulation of UGT2B15 and UGT2B17

In humans, glucuronidation is a critical pathway for the elimination of T and DHT and is mediated by the UDP glucuronosyltransferase (UGT) enzymes [25]. To evaluate mechanisms of regulation of the androgen glucuronidating enzymes UGT2B15 and UGT2B17 by SAFB1, we examined UGT2B15 and UGT2B17 mRNA levels in LNCaP and 22Rv1 cells in response to DHT. DHT repressed UGT2B15 and UGT2B17 mRNA

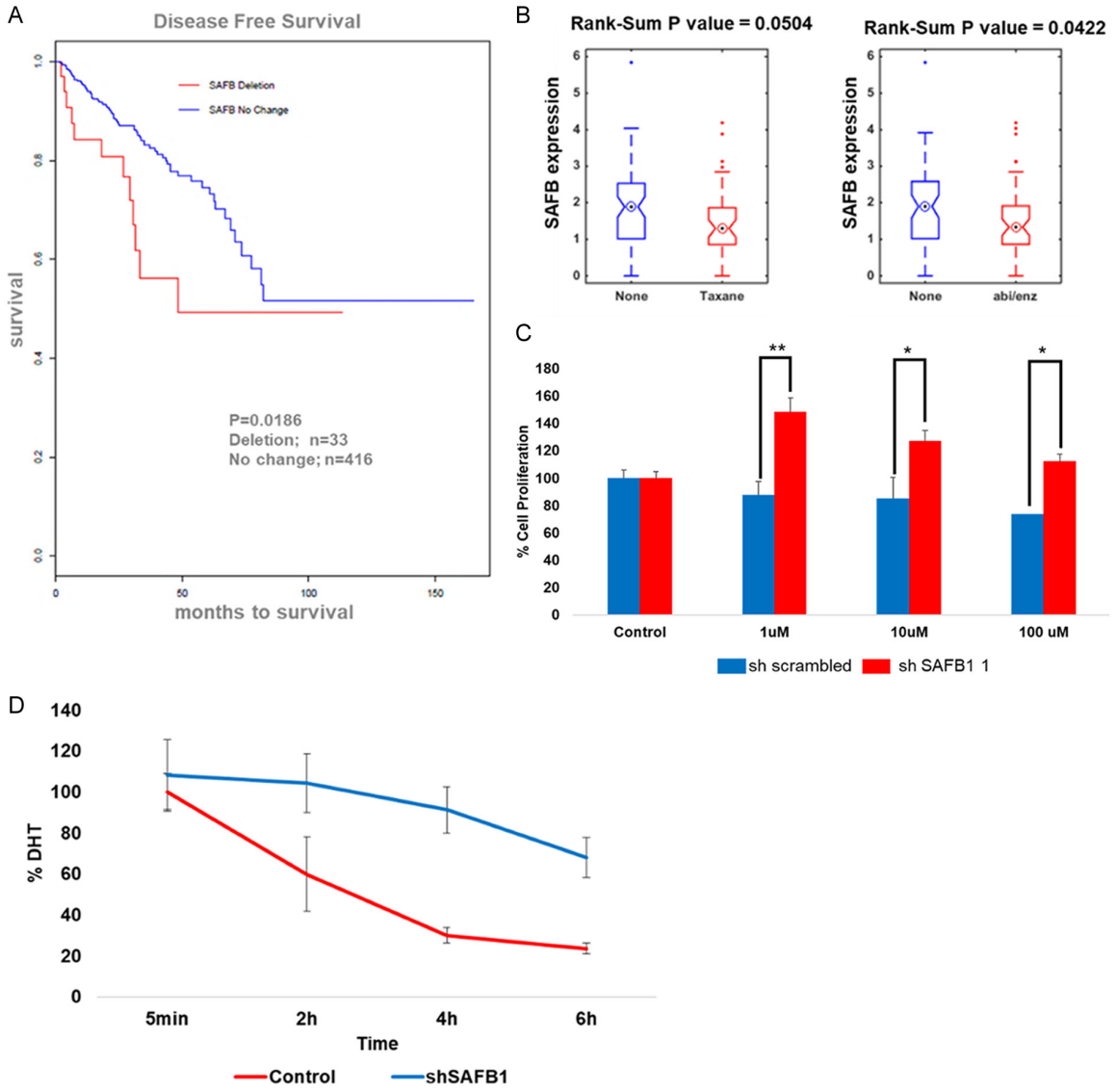

Figure 3. SAFB1 loss has a more aggressive phenotype associated with survival, treatment resistance, and increased androgen stability. (A) Kaplan Meyer log-rank survival curves (n=449) from the TCGA cohorts [22] with respect to the absence or presence of CNA of SAFB1 for patients, no change (n=416) and deletion (n=33). (B) Metastatic PCa patient clinical samples from SU2C/PCF Dream Team cohort [39] were used to measure SAFB1 RNA-seq expression for comparing patients receiving no treatment versus patients receiving Abiraterone and Enzalutamide (Rank-sum *p*-value: 0.0422) or Taxanes (Rank-sum *p*-value =0.0504). (C) Cell proliferation was measured in LNCaP sh control vs sh SAFB1 hp1 was compared after treatment with enzalutamide (control (0 μM), 1 μM, 10 μM, 100 μM) for 4 days. (D) Percent DHT was measured as the change of the collected radiolabeled DHT levels for varying time points (2, 4, 6 HR) measured for the control cells vs the shSAFB1 hp1. For (C), n=3 ± SEM. (*P<0.05, **P<0.005). For (D), n=3 ± SEM.

expression in both cell lines, although the response in the CRPC line 22Rv1 was substantially greater than the hormone-sensitive LNCaP cells (**Figure 4A** and **4B**). Consistent with this, 10 uM enzalutamide treatment upregulated UGT2B15 and UGT2B17 mRNA levels (**Figure 4C**), indicating AR is an direct repressor for these two genes. To identify transcription factors that interact with AR that may regulate these genes, we conducted *in silico* analysis in the online database Swiss Regulon [26]. We identified transcription factors predicted to bind to the UGT2B15 and UGT2B17 proximal promoters within 500 bp to the transcriptional start site. We compared this list of transcription factors to a list of AR interacting proteins

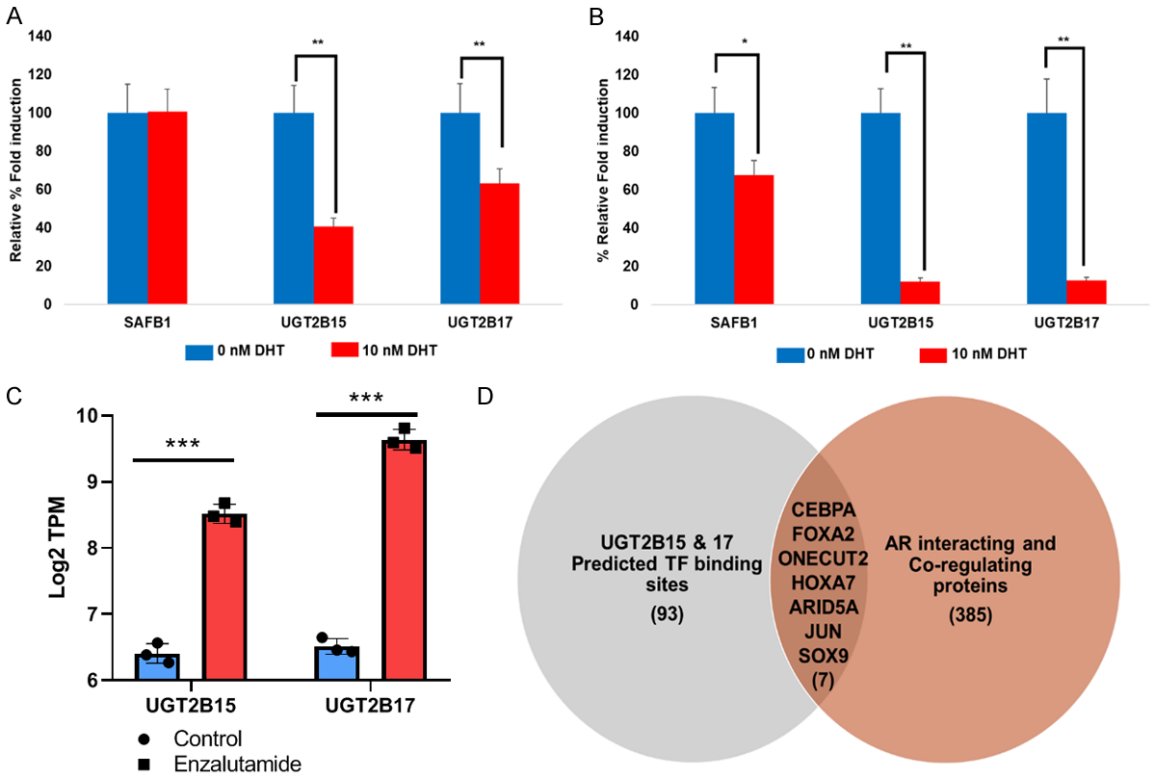

Figure 4. Regulation of UGT2B15 and UGT2B17 genes. (A) Hormone sensitive LNCaP and (B) hormone independent 22Rv1 show significantly repressed UGT2B15 and UGT2B17 mRNA with 10 nM DHT treatment. (C) UGT2B15 and UGT2B17 mRNA expression levels are upregulated with 10 uM Enzalutamide treatment. (D) Analysis of probable AR co-regulatory proteins that may regulate UGT2B15 and UGT2B17 using predicted transcription factor analysis of UGT2B15 and UGT2B17 proximal promoter region and known AR interacting proteins.

curated by McGill University (http://androgendb.mcgill.ca), along with other literature on AR interacting proteins [27-30]. Seven transcription factors (CEBPA, ONECUT2, FOXA2, HOXA7, ARID5A, JUN and SOX9) were identified as interacting with AR and were predicted to bind to the UGT2B15 and/or the UGT2B17 promoter (**Figure 4D**). These are candidate co-regulators of androgen catabolism in concert with SAFB1.

Discussion

This study identified the nuclear matrix protein SAFB1 as a potential regulator of androgen catabolism in PCa through regulation of *UGT2B15* and *UGT2B17* genes, which encode enzymes that inactivate androgens by glucuronidation. We showed previously that the consequences of SAFB1 silencing in LNCaP PCa cells include increased AR transcriptional activity, increased cell proliferation and migration, resistance to anoikis, increased growth in hormone-depleted media, and more rapid tumor growth *in vivo* [9]. Expression microarray profiling of SAFB1-depleted cells revealed that major pathways downregulated following reduction in expression of SAFB1 were glucuronidate metabolic processes, steroid hormone metabolic processes and neuron differentiation. Ranking of the 50 most down-regulated DEGs in SAFB1-depleted cells revealed that the UGT2B gene family was very substantially repressed.

Glucuronidation of T and DHT in humans is the most important catabolic pathway for inactivation and elimination of active androgens [31]. The importance of the role of UGT2B15 and UGT2B17 in regulation of androgen levels was demonstrated by Chouinard et al. [32]. These investigators have shown through transient silencing of UGT2B15 and UGT2B17 concurrently (5-fold mRNA reduction) in LNCaP using siRNA resulted in a marked reduction (75%) of glucuronidated DHT (DHT-G). In prostate cancer cell lines, there was a significant effect of active

androgen metabolite degradation by the reduction of these 2 important enzymes. We predicted that in SAFB1-depleted cells, where UGT2B15 and UGT2B17 mRNA levels are repressed, we would observe a marked increase in stable DHT levels. The measured levels of androgen from the SAFB1 silenced cells showed ~70% of free DHT remaining after 6 h, while in the LNCaP control cells only ~20% of free DHT remained at 6 h. We also observed that the SAFB1-depleted cells, which exhibit the concurrent effects of AR activation as well as an increase in free available DHT, are relatively resistant to the next generation therapeutic compound enzalutamide.

A survey of the regulatory mechanisms of *UGT2B15* and *UGT2B17* described in the literature indicate that AR is a negative regulator of these genes [31, 33-37]. Bao et al. have shown that the effects of AR repression of UGT2B15 and UGT2B17 mRNA expression are significant (70% and 62% reduction) [33]. Inhibitors of AR, flutamide and bicalutamide, were able to cancel out the repressive effects [25, 33]. These investigators also were able to show a direct interaction between AR and the *UGT2B15* and *UGT2B17* promoters using ChIP [34]. These observations demonstrated that androgens negatively regulate their own glucuronidation in PCa cells through AR ligand dependent activation. AR ligand dependent inhibition of UGT2B15 and UGT2B17 expression has been studied as a process that mediates transformation of PCa cells toward a more malignant phenotype. Bao et al. probed AR-positive and AR-negative prostate cancer cell lines for expression of UGT2B15 and UGT2B17 and the AR negative cell lines PC3 and DU145 lacked expression of both genes [34]. These findings suggest AR is also an important positive regulator of the expression of UGT2B15 and UGT2B17, possibly as a feedback mechanism to control androgen levels. However, we showed a major increase in UGT2B15 and UGT2B17 expression in response to enzalutamide, suggesting that the role of regulation of these genes by AR is still not entirely clear.

In summary, we identify SAFB1 to be a mediator of androgen catabolism. This and other findings support an important role for this protein in some forms of PCa where intracrine androgen signaling is physiologically significant. We conclude that SAFB1 expression and activity are pertinent to progression to castration resistance.

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Disclosure of conflict of interest

None.

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