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Original Articles

Androgen deprivation-induced ZBTB46-PTGS1 signaling promotes neuroendocrine differentiation of prostate cancer

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ABSTRACT

Androgen receptor (AR) targeting is an important therapeutic strategy for treating prostate cancer. Most tumors progress to castration-resistant prostate cancer (CRPC) and develop the neuroendocrine (NE) phenotype under androgen deprivation therapy (ADT). The molecular basis for NE transdifferentiation after ADT remains incompletely understood. Herein, we show that an immunocyte expression protein, ZBTB46, induces inflammatory response gene expression and contributes to NE differentiation of prostate cancer cells. We demonstrated a molecular mechanism whereby ZBTB46 can be regulated by the androgen-responsive gene, SPDEF, and is associated with NE prostate cancer (NEPC) differentiation. In addition, ZBTB46 acts as a transcriptional coactivator that binds to the promoter of prostaglandin-endoperoxide synthase 1 (PTGS1) and transcriptionally regulated PTGS1 levels. Overexpression of ZBTB46 decreases the sensitivity of the combination of enzalutamide and a PTGS1 inhibitor; however, knockdown of ZBTB46 sensitizes the PTGS1 inhibitor and reduces tumor malignancy. ZBTB46 is inversely correlated with SPDEF and is increased in higher tumor grades and small-cell NE prostate cancer (SCNC) patients, which are positively associated with PTGS1. Our findings suggest that the induction of ZBTB46 results in increased PTGS1 expression, which is associated with NEPC progression and linked to the dysregulation of the AR-SPDEF pathway.

1. Introduction

The androgen receptor (AR), a nuclear receptor that is activated by binding to androgens [1], promotes the development of prostate cancer [2]. Although traditional AR-targeted therapies are initially effective, most tumors progress to hormone-refractory prostate cancer or castration-resistant prostate cancer (CRPC) within a few years [3]. CRPC patients are commonly treated with androgen deprivation therapy (ADT); however, ADT accelerates neuroendocrine (NE) differentiation with an increased anti-apoptotic stimulus that enables tumor resistance to the AR-targeted therapies [4–7]. NE differentiation is mainly expressed in high-grade and high-stage prostate cancer, particularly in CRPC after ADT [8–11]. An emerging resistant phenotype is NE prostate cancer (NEPC) or small-cell NE prostate cancer (SCNC) characterized by the absence of AR expression or loss of AR signaling [12–14]. The prognosis for NEPC is poor owing to the lack of relevant biomarkers

Abbreviations: ADT, Androgen deprivation therapy; ChIP, Chromatin immunoprecipitation; COX1, Cyclooxygenase 1; CHGA, Chromogranin A; CHGB, Chromogranin B; CRPC, Castration-resistant prostate cancer; CSS, Charcoal-stripped serum; DC, Dendritic cell; EMT, Epithelial-to-mesenchymal transition; ENO2, Enolase 2; GSEA, Gene set enrichment analysis; IF, Immunofluorescence; IHC, Immunohistochemical; NE, Neuroendocrine; NEPC, Neuroendocrine prostate cancer; PTGS1, Prostaglandin-endoperoxide synthase 1; SCNC, Small-cell neuroendocrine prostate cancer; SPDEF, SAM pointed domain containing ETS transcription factor; SYP, Synaptophysin; TCGA, The Cancer Genome Atlas; ZBTB46, Zinc finger and BTB domain containing 46

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and therapeutic approaches that target NEPC patients [7,15]. Increasing attention has been focused on the NE differentiation of prostate cancer [16–19]; however, the mechanisms remain considerably less well understood.

Tumor-associated inflammation is involved in aggressiveness and metastatic progression in several types of cancer [20], including prostate cancer [21,22]. It has been suggested that inducing the expressions of inflammatory response genes may contribute to the development of prostate cancer. Prostaglandin endoperoxide synthase 1 (PTGS1), also known as cyclooxygenase 1 (COX1), was shown to regulate angiogenesis in endothelial cells [23]. Activation of PTGS1 is involved in the inflammatory response, cell proliferation, and fatty acid metabolism during tumor progression [24]. However, the association of abundant PTGS1 with prostate cancer progression and the possible involvement of PTGS1 in AR signaling regulation remain unclear. There is a growing body of evidence linking the inflammatory response to ADT resistance in advanced prostate cancer [25,26]. We hypothesized that the abundance of PTGS1 may be involved in NEPC differentiation following ADT. Therefore, elucidating the regulatory pathway of PTGS1 might lead to a better understanding of the therapeutic targets for NEPC.

We previously identified that a novel prostatic tumor promoter, zinc finger and BTB domain containing 46 (ZBTB46), is negatively regulated by the AR functions via microRNA-1-mediated downregulation [27]. ZBTB46 is a lineage-specific transcription factor that is crucial for different stages of dendritic cell (DC) development [28]. Downregulation of ZBTB46 affects macrophages and DCs in the microenvironment, leading to a decrease in tumor burden [29]. In prostate cancer, ZBTB46 is associated with epithelial-to-mesenchymal transition (EMT) and prostate cancer metastasis by transcriptionally regulating SNAI1 [27]. ZBTB46 overexpression promotes androgen- and AR-independent proliferation [27], which provides insights into the oncogenic role of ZBTB46 to ADT resistance. Although several types of immune responses are induced in prostate cancer following ADT [25,30,31], we hypothesized that the abundance of ZBTB46 is connected to the induction of inflammatory response genes. We investigated the role of ZBTB46 in promoting NEPC progression and its association with PTGS1 expression in ADT-treated prostate cancer.

Our results demonstrated that ADT induces ZBTB46 expression through the downregulation of the androgen-responsive SAM pointed domain containing ETS transcription factor (SPDEF), leading to increased expression of PTGS1 and contributing to NE differentiation of prostate cancer cells. The addition of PTGS1 inhibitor treatment can restore enzalutamide (MDV3100) sensitivity and reduce tumor growth, whereas overexpression of ZBTB46 disrupts the tumor-suppressive effect of this combination treatment and induces PTGS1- and NEPC-associated genes. Our findings provide a novel link between NE differentiation and the expressions of inflammatory response genes as well as new biomarkers and therapeutic targets for NEPC.

2. Materials and methods

2.1. Cell lines and cell culture

The AR-positive C4-2B and LNCaP-AR (parental LNCaP overexpressing wild-type AR [32]) and AR-negative RasB1 (an aggressive cell line expressing a constitutively active Ras in the DU145 cells and isolated from a bone metastasis [27,33–40]) cell lines were obtained from Dr. Kathleen Kelly (NCI/NIH, MD, USA) and maintained as previously described. The PC3, LNCaP, C4-2, and 22Rv1 cell lines were from ATCC (VA, USA), and they were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The NE-like NE-1-8 (a subclone cell from the LNCaP cell line that is treated with long-term ADT [41]) and TRAMP-C1 (a more NE mouse prostate cancer model [42–46]) cell lines were purchased from ATCC and separately cultured in RPMI 1640 medium supplemented with 5% charcoal-stripped serum (CSS) and DMEM medium supplemented with 10% FBS, 0.005 mg/ml bovine insulin (Sigma-Aldrich), and 10 nM dehydroisoandrosterone (Sigma-Aldrich). The AR antagonist, enzalutamide (MDV3100) (Selleck), and the PTGS inhibitor, NS-398 (Sigma-Aldrich), were used separately to treat the cells at 10 μM and 1.77 or 75 μM for 24 h in 10% FBS-containing medium. Dihydrotestosterone (DHT) (Sigma-Aldrich) was used to treat cells at 10 nM for 24 h in 10% CSS-containing medium.

2.2. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using an EZ magna ChIP A kit (Millipore) with a modified protocol. C4-2B and LNCaP-AR cells were treated with DHT (Sigma-Aldrich) at 10 nM for 4 h in 10% CSS-containing medium or MDV3100 (Selleck) at 10 μM for 4 h in 10% FBS-containing medium, as described in Supplementary Methods.

2.3. Promoter reporter assay

For the promoter reporter assays, LNCaP, RasB1, or C4-2B cells in 12-well plates (5×10^4 cells/well) were transiently transfected with 1 μg of the PTGS1- or ZBTB46-green fluorescent protein (GFP) reporter containing ZBTB46- or SPDEF-binding sites. The cells were treated with DHT (Sigma-Aldrich) and MDV3100 (Selleck) or small interfering (si) RNA and DNA (empty vector (EV), ZBTB46- or SPDEF-expressing vector) by transfection, as described in Supplementary Methods.

2.4. Proliferation assay

The cells were stably transfected with a gene encoding an SPDEF or ZBTB46 expression vector or a ZBTB46 or SPDEF short hairpin (sh)RNA vector and seeded at a density of 2000 cells/well in 96-well plates. The cells were treated with MDV3100 at 10 μM and NS-398 at 1.77 or 75 μM for 24 h in 10% FBS-containing medium, as described in Supplementary Methods.

2.5. Colony-formation assay

A colony-formation assay was performed using a starting number of 1000 cells/well seeded in 0.3% agarose (Affymetrix) on top of a 0.6% agarose layer in 12-well plates. Single-cell suspensions of ZBTB46 complementary (c)DNA-transfected 22Rv1 and C4-2B cells, SPDEF/ZBTB46 cDNA-transfected RasB1 cells, or ZBTB46 shRNA vector-transfected PC3 and RasB1 cells were performed as described in Supplementary Methods.

2.6. Tumorigenicity and metastasis assays in mice

The animal work was performed in accordance with a protocol approved by the Taipei Medical University Animal Care and Use Committee (approval No. LAC-2015-0185, Taiwan) as described in Supplemental Materials and Methods. Five-week-old male nude mice were injected with ZBTB46 shRNA vector-transfected RasB1 cells and treated with 20 mg/kg NS-398 or DMSO as the control for 1 month by an intraperitoneal injection twice a day. For the analysis of metastasis, 5-week-old male nude mice (NLAC, Taiwan) were subjected to intracardiac injections of 10^5 cells per mouse of RasB1/EV, RasB1/SPDEF, and RasB1/SPDEF/ZBTB46 cells harboring a luciferase expression vector.

2.7. Immunohistochemical (IHC) staining

We used tissue microarray (TMA) sections, including 16 normal prostatic epithelial samples, 100 primary prostate adenocarcinomas, and eight SCNCs from the Duke University School of Medicine (NC, USA). Twenty-one clinical tissue samples from prostate cancer patients before and after they were treated with ADT were collected from Taipei

Medical University-Wan Fang Hospital (Taiwan). Tissue samples were obtained and used according to the protocols approved by the Duke University School of Medicine-Institutional Review Board (protocol ID: Pro00070193) and the Taipei Medical University-Joint Institutional Review Board (approval No. N201712051). IHC was performed using ZBTB46 (HPA013997, Sigma), SPDEF (TA324209, OriGene), and PTGS1 (ab695, Abcam) antibodies at respective dilutions of 1:250, 1:200, and 1:200, as described in Supplementary Methods.

2.8. Immunofluorescence (IF) staining

TMA sections from the Duke University School of Medicine were rinsed in 2% BSA in PBS for 30 min and then incubated overnight at 4 °C with PTGS1 (ab695, Abcam) and CHGA (ab15160, Abcam) antibodies in 2% BSA/PBS at respective dilutions of 1:200 and 1:150, as described in Supplementary Methods.

2.9. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical calculations were performed with GraphPad Prism analytical tools (GraphPad Software). Differences between individual groups were determined by Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for comparisons among three or more groups. The method for determining the cutoffs was pre-decided by half of the number of patients. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Antagonized AR signaling-induced ZBTB46 exhibited NEPC differentiation

We investigated the role of ZBTB46 in the NE differentiation of prostate cancer cells. We examined the amount of ZBTB46 in a panel of human prostate cancer cells and found that both AR-negative cells (PC3 and RasB1) and the NE-like cell line (NE-1-8) expressed more ZBTB46 and NE markers compared with the AR-positive cells (LNCaP-AR, LNCaP, 22Rv1, and C4-2B) (Fig. 1A and Supplementary Fig. S1A). To examine the abundance of ZBTB46 associated with NEPC differentiation following ADT, we found that long-term treatment with MDV3100 induced ZBTB46 and NE markers, such as chromogranin A (CHGA) and enolase 2 (ENO2) in the AR-positive cells (22Rv1, C4-2, C4-2B, and LNCaP) (Fig. 1B–E). Our results support the stimulation of NE transformation of prostate cancer with continued ADT treatment [8–11,47]. Moreover, our results show that the CRPC C4-2 and 22Rv1 cells responded better to the MDV3100 than the androgen-dependent LNCaP cells (Fig. 1B and D), consistent with the studies showing that androgen-independent cases have better NE differentiation than the androgen-dependent cases [48–50]. Overexpression of ZBTB46 induced an abundance of NE markers in the 22Rv1 and C4-2B cells (Fig. 1F and G). In contrast, ZBTB46-knockdown decreased the level of NE markers in the RasB1 and PC3 cells (Fig. 1H and I, left), and similar results were found in the NE-1-8 cells (Fig. 1I, right). We further performed ZBTB46-knockdown in the C4-2B cells and treated the cells with MDV3100. The results showed that ZBTB46-knockdown reduced NE markers regardless of MDV3100 treatment (Fig. 1J), confirming that ZBTB46 regulates NEPC differentiation. Moreover, we found that in addition to the NE markers (CHGA, chromogranin B (CHGB), and ENO2) being reduced, the reprogramming transcription factor, SOX2, and a key factor in the EMT (SNAI1), also decreased in the ZBTB46-knockdown NE-1-8 cells and mouse TRAMP-C1 cells (Supplementary Fig. S1B). These results suggest that ZBTB46 induction is associated with NEPC differentiation.

3.2. SPDEF transcriptionally represses ZBTB46 in prostate cancer cells

Unlike luminal-type tumor cells, NE cells do not express AR, and AR signaling is repressed [12]. We next validated ZBTB46 expression in the Cancer Genome Atlas (TCGA) prostate dataset by a gene set enrichment analysis (GSEA) with the gene signatures that reflect AR signaling components [51,52]. We found that the tissues expressing low levels of ZBTB46 were more likely to have gene signatures associated with activated androgen-responsive signatures (Supplementary Fig. S2A). We identified SPDEF as a potential regulator of ZBTB46 in the core enrichment group (Supplementary Fig. S2B). SPDEF is a critical tumor suppressor in prostate cancer, and it is highly expressed in normal prostate epithelial cells [40]. To further confirm the inverse association between SPDEF and ZBTB46, we found that knockdown of SPDEF in the AR-positive cells induced ZBTB46 mRNA expression (Fig. 2A), whereas the SPDEF-overexpressing AR-negative cells had significantly lower ZBTB46 mRNA levels (Fig. 2B). In addition, SPDEF could directly regulate *ZBTB46* by transcriptional function. Based on our ChIP-Seq data analyzed from a published dataset (GSE48930) [53], SPDEF may be physically associated with the *ZBTB46* promoter (Supplementary Fig. S2C). We hypothesized that SPDEF transcriptionally represses *ZBTB46* in prostate cancer cells by directly binding to the SPDEF response element (SRE) on the *ZBTB46* promoter. We next looked for sequences resembling the putative SRE in the *ZBTB46* promoter region and found 10 SREs located between −3 and −1 kb upstream of the transcriptional start site (Fig. 2C). The ChIP results confirmed that the most-enriched ChIP product was found at the putative SPDEF-binding sites, SRE2 and SRE3/4 (Fig. 2D). Moreover, the SRE2 and SRE3/4 sites were found to have increased SPDEF binding in response to DHT (Fig. 2E), but this binding was reduced in the cells after MDV3100 treatment (Supplementary Fig. S2D). We next performed reporter assays with serially deleted promoter constructs containing SREs (Fig. 2C) and found that E2 and E3 reporters had the lowest reporter activities compared with the E1 reporter (Fig. 2F). Wild-type (WT) E2 and E3 reporter activities were reduced in the presence of DHT (Fig. 2G), whereas E2 or E3 mutants did not change (Fig. 2H). Moreover, decreased reporter gene activities were detected when WT E2 and E3 reporter constructs were cotransfected with SPDEF expression vector in RasB1 cells, whereas E2 and E3 mutants disrupted the repressive ability of SPDEF (Fig. 2I). These data are consistent with a mechanism whereby SPDEF represses *ZBTB46* transcription by directly and physically interacting with the *ZBTB46* promoter.

3.3. Loss of SPDEF is associated with NE differentiation of prostate cancer cells

We next analyzed the relationship between SPDEF and ZBTB46 in a panel of prostate cancer cells and found an inverse relationship between SPDEF and ZBTB46 (Fig. 3A). These results were confirmed in the prostate cancer cells treated with DHT or MDV3100. As expected, DHT increased SPDEF abundance and ZBTB46 reduction, whereas the treatment with MDV3100 resulted in a decrease in SPDEF and an increase in ZBTB46 in the AR-positive cells (Fig. 3B, left and middle). These results were confirmed in the AR-negative cells, which showed nonsignificant changes in SPDEF and ZBTB46 following perturbation of AR signaling (Fig. 3B, right). We next examined whether ZBTB46 abundance was affected by SPDEF, which is involved in NEPC differentiation. We found that the NE markers (CHGA, CHGB, and ENO2) increased in the LNCaP and C4-2B cells following SPDEF-knockdown (Fig. 3C). These results were confirmed by Western blots, which showed that the SPDEF protein decreased and that the ZBTB46 and ENO2 proteins increased following SPDEF-knockdown (Fig. 3D). Moreover, induction of ZBTB46 and ENO2 proteins was observed in the C4-2B cells in the presence of SPDEF shRNA regardless of DHT treatment (Fig. 3E). We also found that SPDEF overexpression decreased the protein levels of endogenous ZBTB46 and ENO2 even in the cells

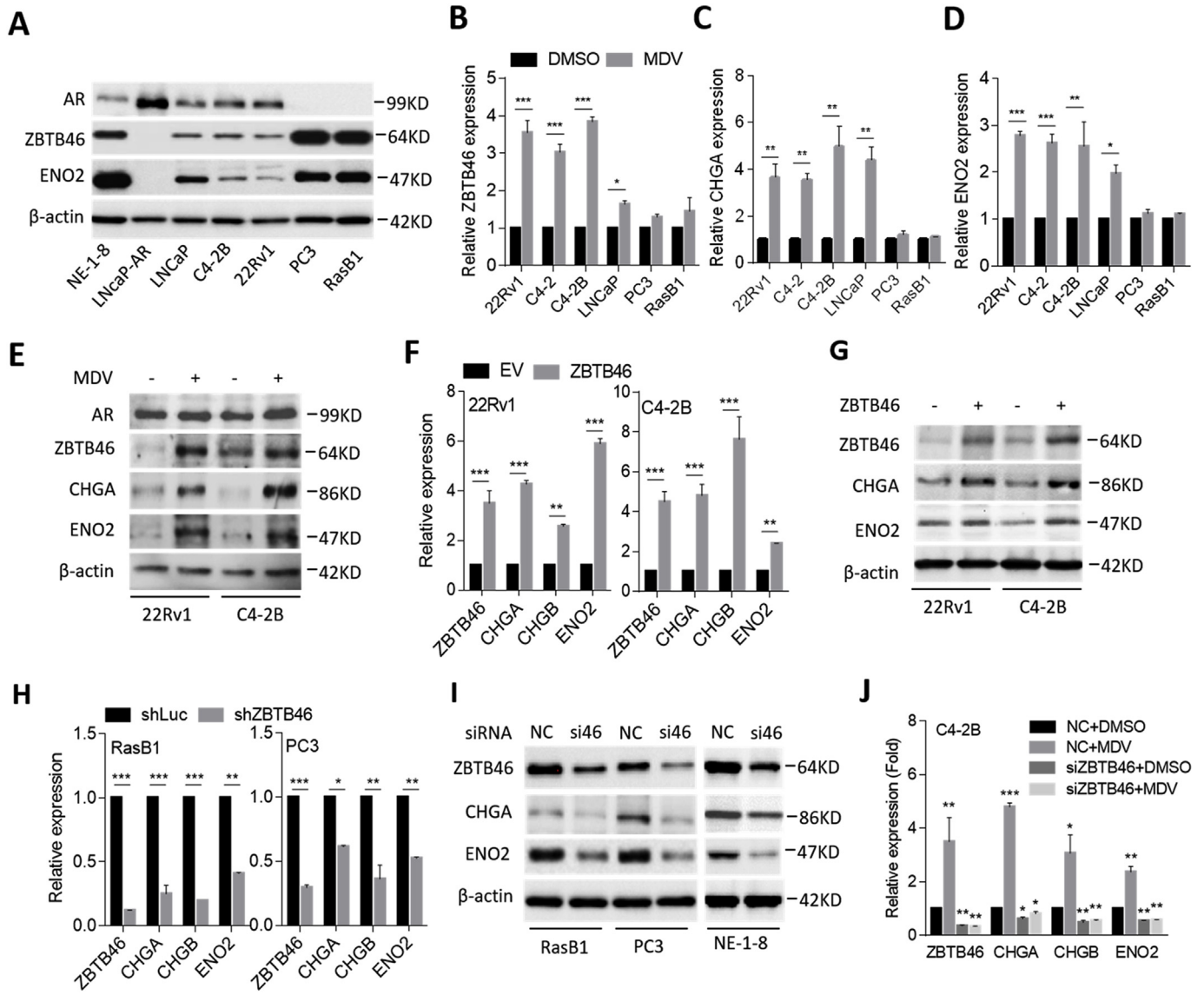

Fig. 1. ZBTB46 induces NE marker expressions of prostate cancer. (A) Immunoblotting for AR, ZBTB46, and ENO2 in various prostate cancer cell lines. (B–D) ZBTB46, CHGA, and ENO2 mRNA levels after MDV3100 treatment at 10 μM in FBS-containing medium for 2 weeks of various prostate cancer cell lines. (E) Immunoblotting of extracts from 22Rv1 and C4-2B cells following MDV3100 treatment for 2 weeks. (F) ZBTB46 and NE markers (CHGA, CHGB, and ENO2) in 22Rv1 and C4-2B cells following transient transfection with ZBTB46 or an empty vector (EV) by qRT-PCR. (G) ZBTB46, CHGA, and ENO2 proteins in 22Rv1 and C4-2B cells following stable transfection with EV and ZBTB46 expression vectors. (H) ZBTB46 and NE markers in RasB1 and PC3 cells stably expressing ZBTB46 shRNA or control vector (shLuc) by qRT-PCR. (I) Immunoblots showing ZBTB46, CHGA, and ENO2 in RasB1, PC3, and NE-1-8 cells following control (NC) or ZBTB46 siRNA (si46) transfection. (J) ZBTB46 and NE markers (CHGA, CHGB, and ENO2) in C4-2B cells transiently transfected with NC or ZBTB46 siRNA and then treated with MDV3100 in FBS-containing medium for 24 h by qRT-PCR. The quantification results of mRNA are presented as the mean ± SEM, $n = 3$ biological replicates. Significance was determined by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

treated with MDV3100 (Fig. 3F). We then analyzed the ZBTB46 and NE markers after SPDEF overexpression in PC3 and RasB1 cells and mouse TRAMP-C1 cells. We found that ectopic SPDEF significantly decreased the levels of ZBTB46- and NEPC-associated genes (Fig. 3G and H and Supplementary Fig. S3A). These results verified a model whereby inhibition of the AR mediates a decrease in SPDEF, leading to the induction of ZBTB46- and NEPC-associated genes of prostate cancer cells.

3.4. ZBTB46 overcomes the tumor suppressor effects of SPDEF in prostate cancer

To assess the contribution of SPDEF to the antitumor effect of prostate tumorigenesis, we found that SPDEF overexpression reduced RasB1 cell proliferation and colony formation compared with the cells carrying an empty vector (EV) (Fig. 4A and B). We next examined the functional relevance of ZBTB46 and found that ZBTB46 overexpression induced the cell proliferation rate of SPDEF-expressing RasB1 cells (Fig. 4A and B). Because C4-2B expressed abundant SPDEF (Fig. 3A), the results showed no significantly reduced cell proliferation rate or invasion potential in the SPDEF-overexpressing C4-2B cells (Supplementary Figs. S3B and C). In addition, we rescued ZBTB46 in the SPDEF-overexpressing LNCaP cells. As expected, SPDEF did not decrease the cell growth rates in the LNCaP cells; however, the ZBTB46-rescued cells exhibited increased cell proliferation (Supplementary Fig. S3D). We also found that ZBTB46 promoted SPDEF-overexpressing LNCaP cell proliferation regardless of MDV3100 treatment (Supplementary Fig. S3E), demonstrating that ZBTB46 bypasses the tumor-suppressive effect of SPDEF. The SPDEF and ZBTB46 levels were confirmed by immunoblotting or qRT-PCR from these cells by manipulating the SPDEF and ZBTB46 expressions (Fig. 4C and Supplementary

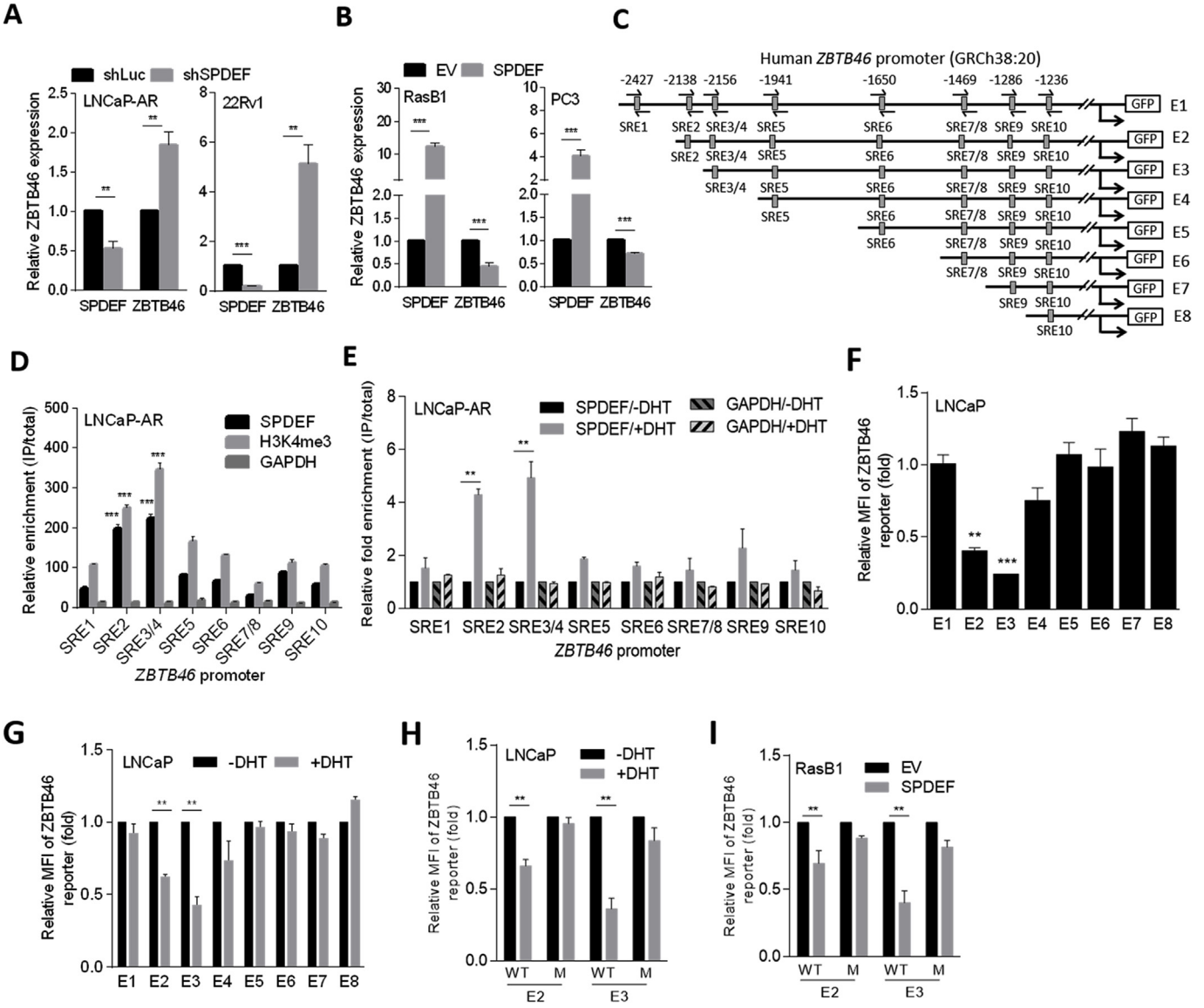

Fig. 2. Repression of ZBTB46 by SPDEF-dependent transcriptional regulation. (A) SPDEF and ZBTB46 mRNA levels in LNCaP-AR and 22Rv1 cells stably expressing an SPDEF shRNA or control vector (shLuc). (B) SPDEF and ZBTB46 levels in RasB1 and PC3 cells following stable transfection with SPDEF or a control vector (empty vector (EV)) by qRT-PCR. (C) Schematic of the predicted SPDEF responsive elements (SREs) in serially deleted promoter green fluorescent protein (GFP)-reporter constructs of human *ZBTB46*. (D) ChIP assays of LNCaP-AR cells with antibodies against SPDEF, H3K4me3, and GAPDH. * vs. SRE1. (E) ChIP assays in LNCaP-AR cells treated with DHT. Enrichment is given as a percentage of the total input and then normalized to IgG. (F) Relative median fluorescent intensities (MFIs) of ZBTB46 reporters (E1-E8) in LNCaP cells. (G) The same assay as in 2F in LNCaP cells cultured in charcoal-stripped serum (CSS)-containing medium and cells treated with DHT. (H) Relative MFIs of wild-type and mutant-ZBTB46 reporters (E2 and E3) in response to DHT in LNCaP cells. (I) Relative MFIs of wild-type and mutant-ZBTB46 reporters (E2 and E3) in response to SPDEF overexpression in RasB1 cells. Quantification of mRNA, ChIP data, and MFIs are given as the mean ± SEM of three independent experiments. Significance was determined by Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

Fig. S3F). Moreover, the mice administered a subcutaneous injection of SPDEF-expressing RasB1 cells showed significantly decreased tumor sizes and weights compared with the mice injected with cells harboring the EV (Fig. 4D–F), confirming the tumor-suppressive role of SPDEF. However, the mice injected with ZBTB46-expressing RasB1/SPDEF cells showed increased tumor growth and tumor weights (Fig. 4D–F). We further tested whether ZBTB46 affects NEPC differentiation in these injected tumors by IHC staining. The results showed that ZBTB46 and the NE marker synaptophysin (SYP) increased in tumors from the mice injected with ZBTB46-expressing RasB1/SPDEF cells compared with the mice injected with RasB1/SPDEF cells (Fig. 4G and H). These data demonstrate that ZBTB46 induction is associated with NEPC differentiation in SPDEF-expressing cells. In addition, we monitored the metastatic functions of ZBTB46 and found that the mice receiving an intracardiac injection of ZBTB46-expressing RasB1/SPDEF cells had a lower survival rate (Fig. 4I) and increased incidences of bone metastasis (Fig. 4J and K) compared with the mice receiving an injection of RasB1/SPDEF cells. These results support our hypothesis that the abundance of ZBTB46 contributes to NE differentiation and induces bone metastasis of prostate cancer.

3.5. *ZBTB46 is positively associated with PTGS1 in response to inhibition of AR signaling*

ZBTB46 is highly expressed in dendritic cells (DCs), and it acts as a transcription factor selectively expressed by classical DCs and their committed progenitors [54]. We hypothesized that the induction of ZBTB46 is associated with inflammatory responses in prostate cancer.

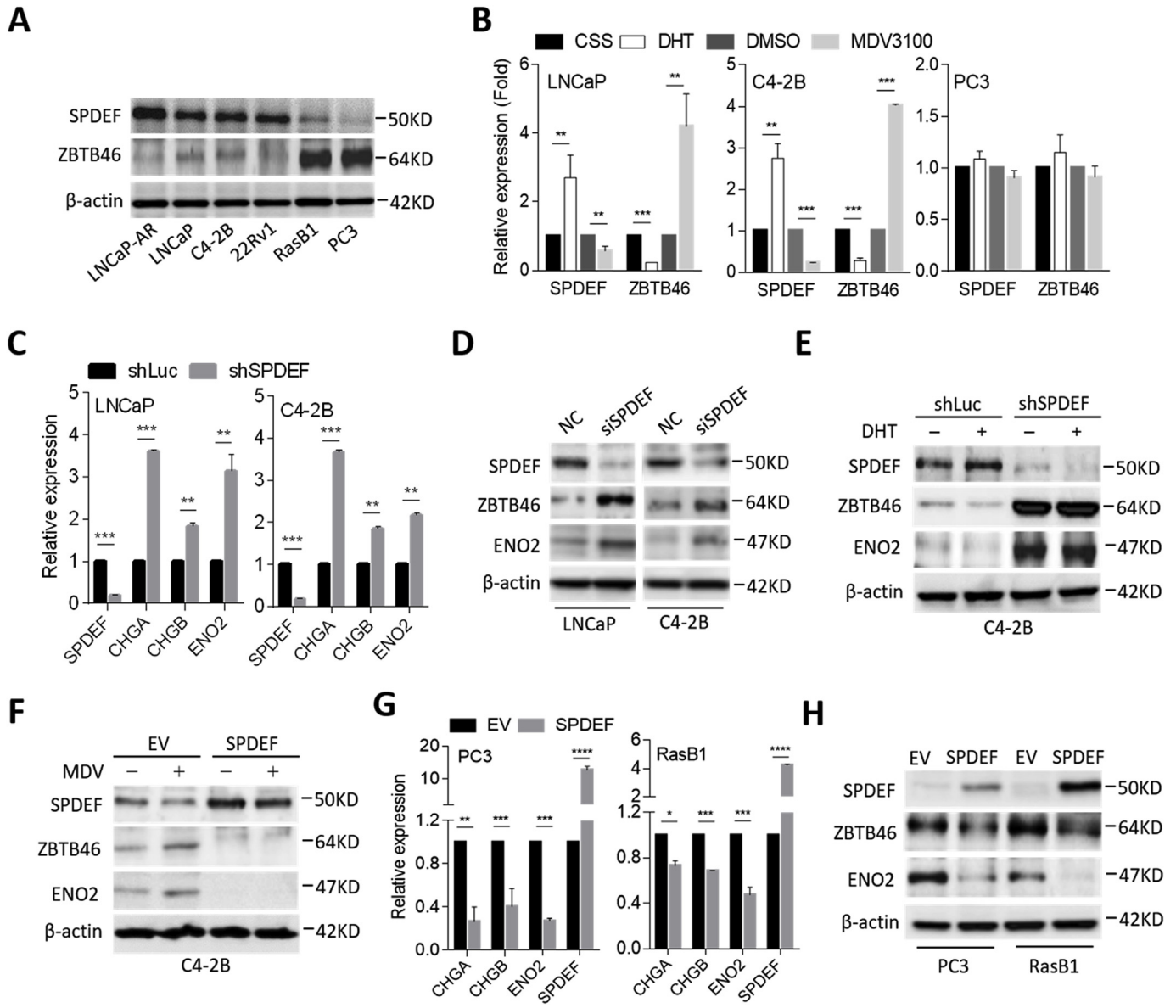

Fig. 3. Reduced SPDEF is essential for ZBTB46 expression and involved in NEPC differentiation. (A) Western blotting of SPDEF and ZBTB46 in various prostate cancer cell lines. (B) SPDEF and ZBTB46 mRNA levels in LNCaP, C4-2B, and PC3 cells after 24 h of treatment with DHT or MDV3100 relative to the controls (charcoal-stripped serum (CSS) or DMSO, respectively). (C) RNA levels of SPDEF and NE markers (CHGA, CHGB, and ENO2) in LNCaP and C4-2B cells stably expressing an SPDEF shRNA or control vector (shLuc). (D) SPDEF, ZBTB46, and ENO2 proteins as determined by immunoblotting in LNCaP and C4-2B cells following the control (NC) or SPDEF siRNA transfection. (E) SPDEF, ZBTB46, and ENO2 proteins in C4-2B cells stably expressing an SPDEF or Luc shRNA vector and cells treated with DHT in CSS-containing medium for 24 h. (F) Western blotting of SPDEF, ZBTB46, and ENO2 in C4-2B cells following stable empty vector (EV) or SPDEF expression and then treatment with MDV3100 in FBS-containing medium for 24 h (G) NE markers (CHGA, CHGB, and ENO2) and SPDEF in PC3 and RasB1 cells following stable transfection with an EV or SPDEF by qRT-PCR. (H) SPDEF, ZBTB46, and ENO2 proteins as determined by immunoblotting of PC3 and RasB1 cells following stable EV or SPDEF expression. Quantification of mRNA is presented as the mean ± SEM, $n = 3$ biological replicates. Significance was determined by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Because PTGS is key to the inflammatory response in cancers [23,24], we investigated the role of PTGS in ZBTB46-promoted malignant progression. We measured the ZBTB46, PTGS1, and PTGS2 expressions in AR-positive cells relative to perturbations in AR signaling. We found that the DHT-treated C4-2B and 22Rv1 cells had lower ZBTB46 and PTGS1 expressions, whereas PTGS2 expression was not affected by the DHT treatment (Fig. 5A). Moreover, the ZBTB46 and PTGS1 expressions increased when the cells were treated with MDV3100 (Fig. 5B). We further analyzed the PTGS1 and PTGS2 proteins in the ectopic ZBTB46-expressing AR-positive cells in response to DHT. Indeed, DHT reduced endogenous ZBTB46 and PTGS1 expressions, whereas ZBTB46 overexpression induced PTGS1, but not PTGS2, despite DHT treatment (Fig. 5C). The GSEAs of TCGA prostate cancer dataset showed that ZBTB46 induction was positively associated with the expressions of gene sets that were activated by inflammatory response pathways (Gene Ontology and BioCarta) (Supplementary Fig. S4A). Significantly, the GSEAs showed that the tissues expressing low PTGS1 but not PTGS2 were associated with upregulated androgen-responsive signatures [51,52] (Supplementary Fig. S4B). These data suggest a negative association between PTGS1 expression and androgen-responsive signaling activation. We hypothesized that antagonized AR signaling-induced ZBTB46 acts as a transcriptional activator of *PTGS1*. Notably, ZBTB46-knockdown in RasB1 and PC3 cells reduced PTGS1 mRNA expression (Fig. 5D). In contrast, ZBTB46-overexpressing 22Rv1 and C4-2B cells

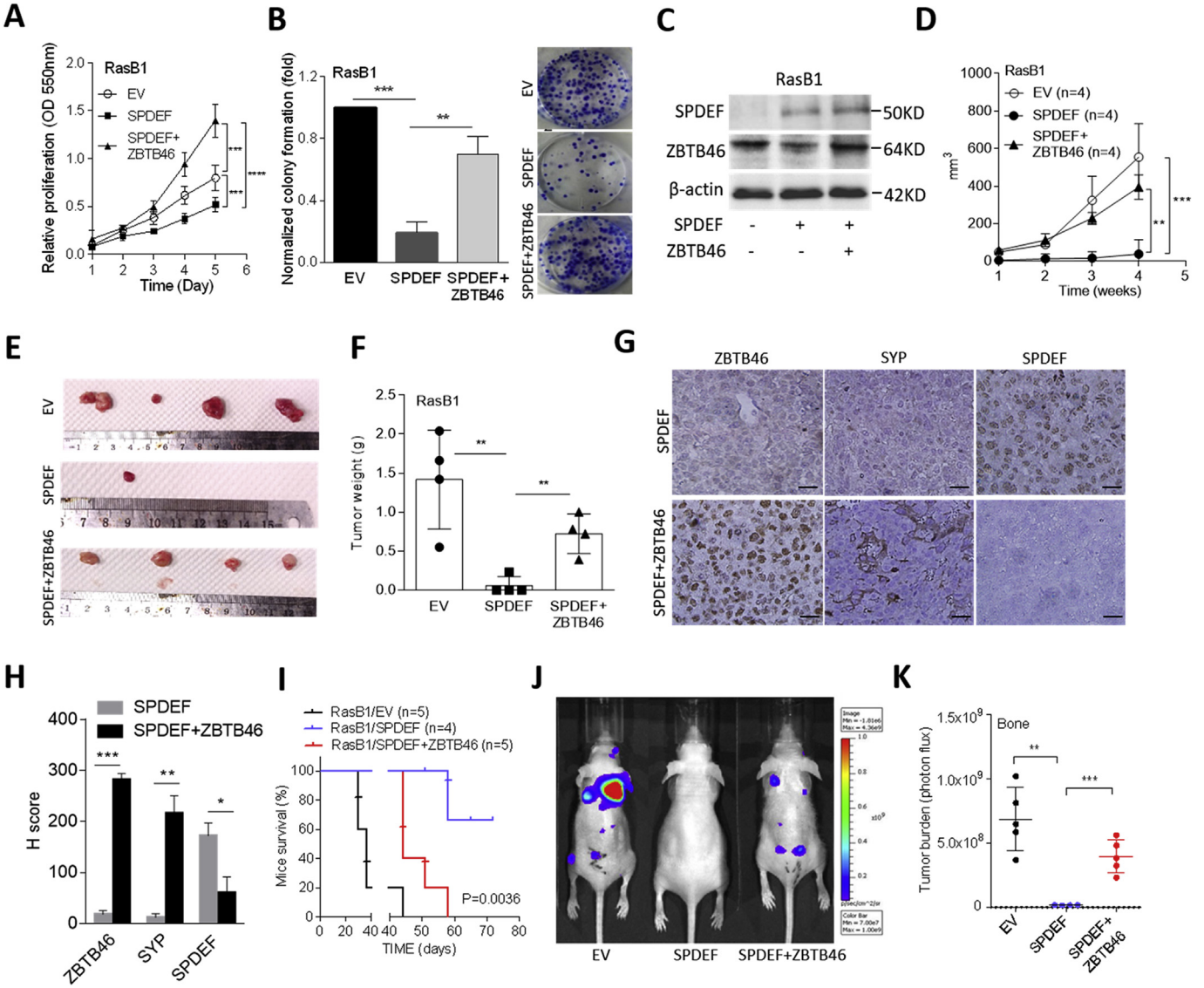

Fig. 4. ZBTB46 bypasses the tumor-suppressive effect of SPDEF. (A) Proliferation of RasB1 cells transfected with an empty vector (EV), SPDEF, or SPDEF + ZBTB46; $n = 8$. (B) Quantification ($n = 3$, left) and selected images (right) of colony-formation assays of the stable RasB1 cell line containing an EV, SPDEF, or SPDEF + ZBTB46. (C) Western blotting of samples from cells assessed in 4A and 4B. (D–F) Growth (D), images (E), and weights (F) of tumor xenografts in male nude mice 4 weeks after subcutaneous inoculation with RasB1 cells stably expressing an EV, SPDEF, or SPDEF + ZBTB46. $n = 4$ mice per group. (G and H) IHC staining (G) and analysis (H) of subcutaneous tumors with antibodies specific for ZBTB46, SYP, and SPDEF in tumor-bearing mice from 4E. Scale bars represent 50 μm. (I–K) Survival analysis by a log-rank test (I) and bioluminescence imaging analysis (J and K) of prostate tumor cell lesions in the bone of mice 45 days after receiving an intracardiac injection of RasB1 cells stably transfected with the EV ($n = 5$), SPDEF ($n = 4$), or SPDEF + ZBTB46 ($n = 5$). Quantification of the proliferation and colony formation assays is presented as the mean ± SEM from three biological replicates. Significance was determined by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

had significantly higher PTGS1 mRNA expressions (Fig. 5E). The analysis of putative ZBTB46 response elements (ZREs) [55] identified a candidate ZRE in the *PTGS1* promoter (Fig. 5F). The ChIP assays indicated that the ZRE site was enriched with antibodies against ZBTB46 and a positive control H3K4me3 (Fig. 5G). We also found significantly decreased ZBTB46 binding at the putative ZRE after the DHT treatment (Fig. 5H, left), whereas endogenous binding of ZBTB46 was induced after the MDV3100 treatment (Fig. 5H, right). Using a reporter assay, we found that DHT decreased WT reporter activity, but the MDV3100 treatment induced reporter activity (Fig. 5I). Moreover, increased reporter activity was detected in the cells with ZBTB46 overexpression, whereas ZBTB46-knockdown reduced reporter activity (Fig. 5J). We mutated the putative ZRE in the *PTGS1* promoter (Fig. 5F) and found that mutating ZRE disrupted reporter activity by either the inhibitory effects of DHT and ZBTB46 siRNA or the stimulating effects of MDV3100 and ZBTB46-expressing vector (Fig. 5I and J). We concluded that PTGS1 induction by ADT is dependent on ZBTB46-upregulated transcriptional activation. These data support a positive association between ZBTB46 and PTGS1 in prostate cancer cells after ADT.

3.6. Ectopic ZBTB46 reduces the sensitivity of the combination of MDV3100 and a PTGS1 inhibitor

To study the effect of the anti-inflammatory drug on prostate cancer after ADT, we assessed the contribution of a PTGS inhibitor to re-sensitized anti-AR therapy in prostate cancer cells. We used a PTGS inhibitor (NS-398) alone or in combination with MDV3100 to treat C4-2B and 22Rv1 cells. We found no significant reduction in the cell morphology and growth rate with NS-398 treatment alone specific to either PTGS1 (75 μM) or PTGS2 (1.77 μM) (Supplementary Figs. S5A

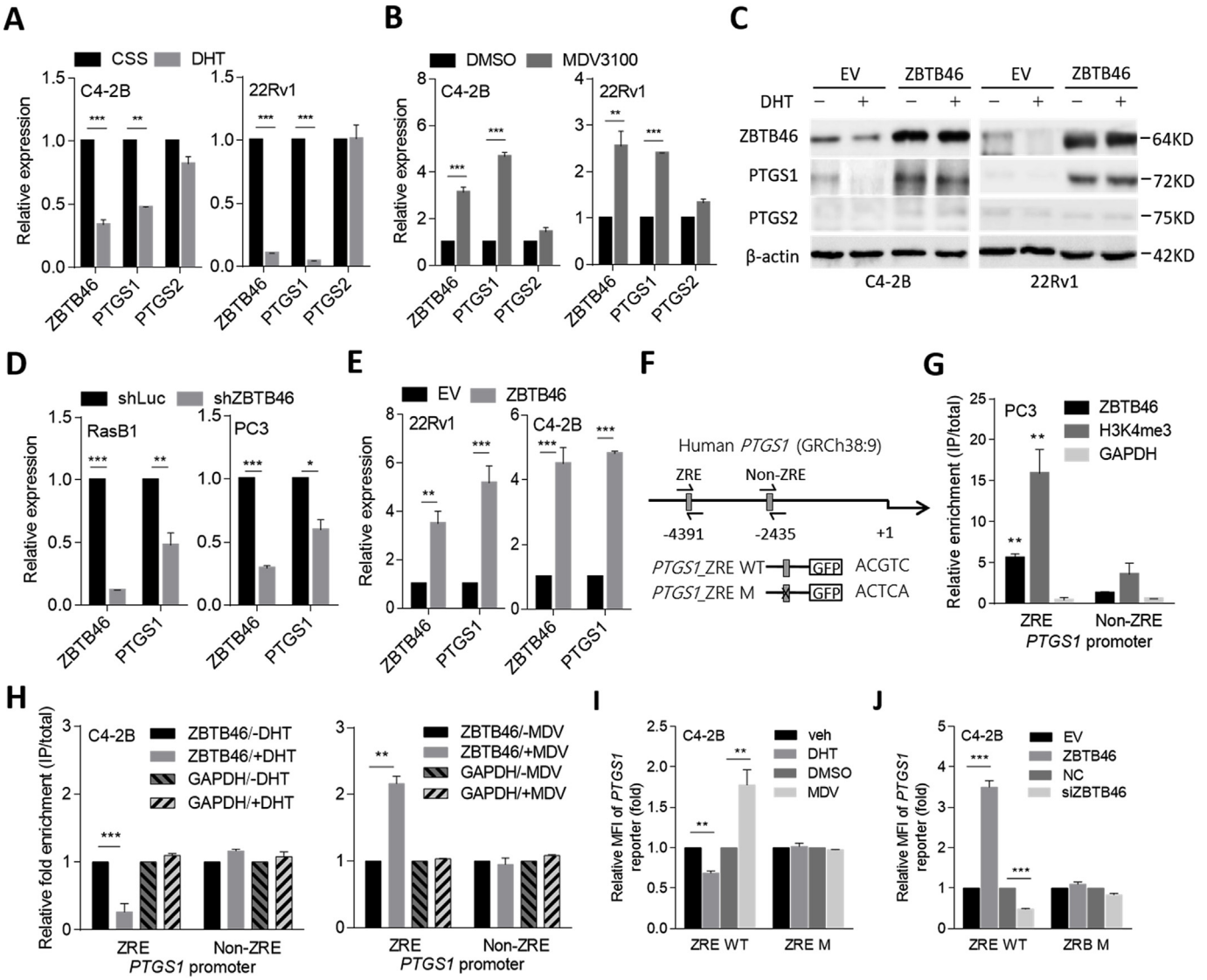

Fig. 5. Positive association between ZBTB46 and PTGS1 in prostate cancer cells in response to AR signaling. (A and B) ZBTB46, PTGS1, and PTGS2 mRNA levels in C4-2B and 22Rv1 cells treated with DHT in charcoal-stripped serum (CSS)-containing medium (A) or MDV3100 in FBS-containing medium (B) for 24 h. (C) Western blotting of ZBTB46, PTGS1, and PTGS2 in C4-2B and 22Rv1 cells transiently transfected with an empty vector (EV) or ZBTB46 expression vector for 24 h and then treated with DHT in CSS-containing medium for 24 h (D) RNA levels of ZBTB46 and PTGS1 in RasB1 and PC3 cells stably expressing a ZBTB46 shRNA or control vector (shLuc). (E) ZBTB46 and PTGS1 mRNAs as determined by qRT-PCR in 22Rv1 and C4-2B cells following control (EV) or ZBTB46 expression. (F) Schematic of the predicted ZBTB46 responsive element (ZRE), the non-ZBTB46 responsive element (Non-ZRE), and the ZRE wild-type (WT) and mutant (M) promoter green fluorescent protein (GFP)-reporter constructs of human *PTGS1*. (G) ChIP assays of PC3 cells with antibodies against ZBTB46, H3K4me3, and GAPDH. Enrichment is given as a percentage of the total input and then normalized to IgG. * vs. non-ZRE. (H) ChIP assays in C4-2B cells treated with DHT (left) or MDV3100 (right) with antibodies against ZBTB46 and GAPDH. (I and J) Relative mean fluorescent intensities (MFIs) of PTGS1 reporters (ZRE WT and ZRE M) in C4-2B cells treated with DHT or MDV3100 (I) or transfected with the ZBTB46 expression vector or siRNA (J). The quantification of mRNA, ChIP data, and MFIs is presented as the mean ± SEM from three independent experiments. Significance was determined by Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

and B and Fig. 6A). Interestingly, the combination of the MDV3100 and NS-398 treatment with only concentration-specific PTGS1 reduced tumor growth (Fig. 6A). We also found that this combined treatment reduced ZBTB46 expression but not in the C4-2B and 22Rv1 cells treated with MDV3100 or NS-398 alone (Fig. 6B). The AR-negative RasB1 cells did not respond to this combined treatment (Supplementary Figs. S5C and D). These data suggest that specific inhibition of PTGS1 can induce sensitivity to MDV3100 and reduce ZBTB46 expression. Moreover, with ectopic ZBTB46 expression, the C4-2B and 22Rv1 cells showed increased cell proliferation (Supplementary Fig. S5E) and colony formation (Fig. 6C and Supplementary Fig. S5F) despite treatment with a combination of MDV3100 and NS-398. We also found that the ZBTB46-overexpressing C4-2B cells induced PTGS1 and ENO2 abundance despite this combined treatment (Fig. 6D), demonstrating that high ZBTB46 expression led to PTGS1 induction and NE differentiation of the prostate cancer cells. We further explored the tumor growth effect of ZBTB46 in the NS-398-treated cells. We found that the PC3 and RasB1 cells with ZBTB46-knockdown (Supplementary Fig. S4G) showed reduced cell proliferation and colony formation and further reductions following NS-398 treatment (Fig. 6E and F and Supplementary Figs. S5H and I). The tumor-formation capability of RasB1 cells with ZBTB46-knockdown was further tested by a subcutaneous injection. The results show that the mice injected with the RasB1 cells expressing control shRNA did not exhibit reduced tumor growth rates after the NS-398 treatment; however, the mice injected with the ZBTB46-knockdown cells and treated with NS-398 exhibited reduced tumor sizes and weights (Fig. 6G–I). These results indicate that the ZBTB46 levels may have affected the sensitivity to the anti-

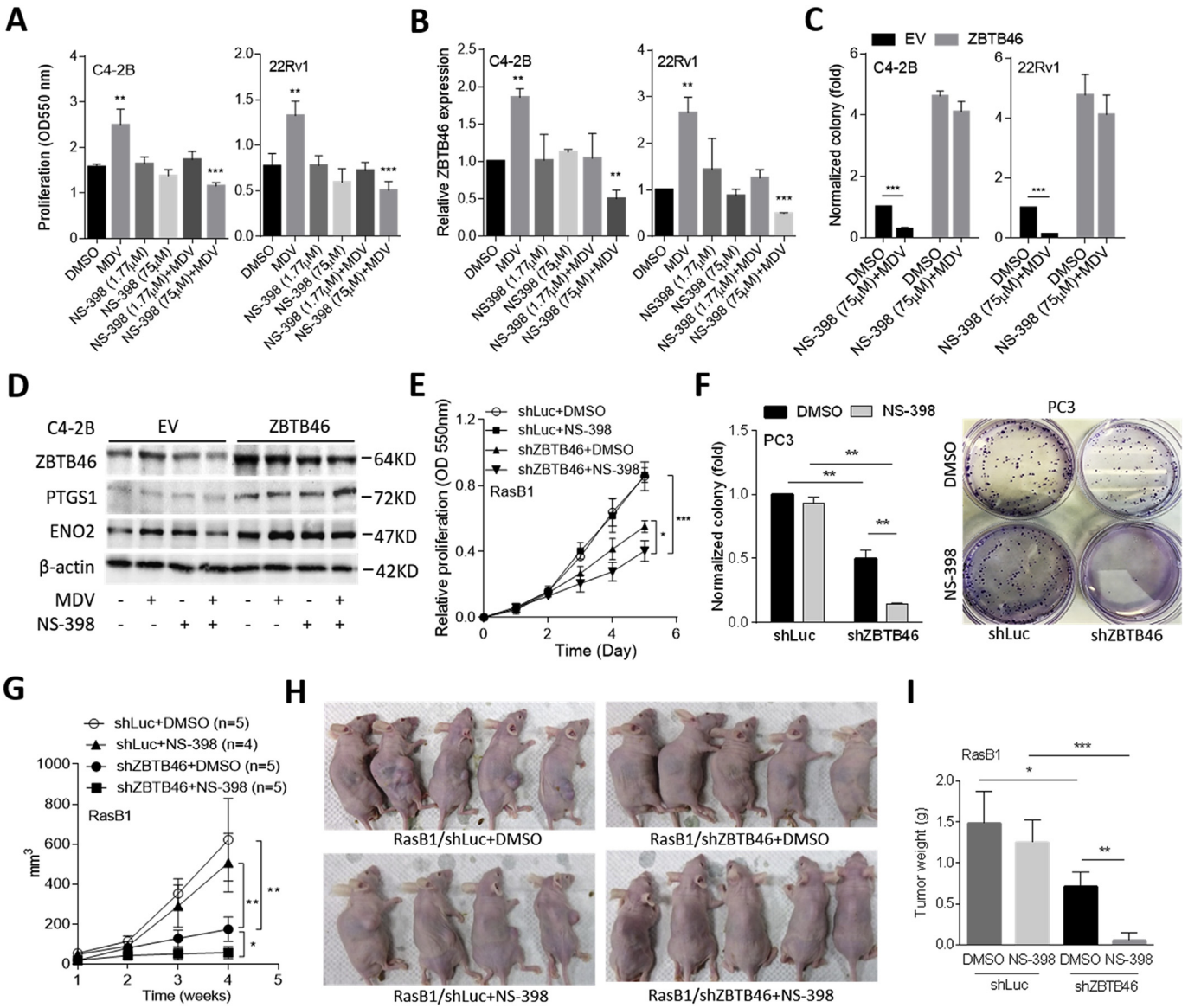

Fig. 6. ZBTB46 regulates the sensitivity of the PTGS1 inhibitor and induces tumor growth. (A) Proliferation assay in C4-2B and 22Rv1 cells treated with enzalutamide (MDV3100, 10 μM) and a PTGS inhibitor (NS-398, 1.77 and 75 μM), $n = 8$. DMSO, control. * vs. DMSO. (B) Monitoring of ZBTB46 mRNAs in C4-2B and 22Rv1 cells following treatment with MDV3100 and NS-398. * vs. DMSO. (C) Quantification of the colony formation of C4-2B and 22Rv1 cells with combined MDV3100 and NS-398 treatment following ZBTB46 or empty vector (EV) overexpression. (D) Western blotting of ZBTB46, PTGS1, and ENO2 in C4-2B cells treated with MDV3100 and NS-398 following stable EV or ZBTB46-expressing vector transfection. (E) Proliferation assays in RasB1 cells treated with a PTGS1 inhibitor (NS-398, 75 μM) following stable ZBTB46 or control (Luc) shRNA vector expression, $n = 8$. DMSO, control. (F) Quantification and images of the colony formation of PC3 cells treated with NS-398 following ZBTB46 or Luc shRNA vector overexpression. (G–I) Tumor growth analysis of stable RasB1 cell lines (shLuc vs. shZBTB46) subcutaneously inoculated into male nude mice followed by treatment with NS-398. Tumor sizes were monitored weekly (G), and images (H) and tumor weights (I) were also measured at the end. $n = 5$ mice per group. Quantification of the mRNA, proliferation, and colony formation assays is presented as the mean ± SEM from three biological replicates. Significance was determined by Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

inflammatory drug.

3.7. ZBTB46 is inversely correlated with SPDEF and positively correlated with PTGS1 in prostate cancer patients receiving ADT

To study the relationships among ZBTB46, SPDEF, and PTGS1, we analyzed a prostate cancer TMA collected from the Department of Pathology, Duke University School of Medicine (NC, USA). The IHC results showed a reverse correlation between ZBTB46 and SPDEF and a positive correlation between ZBTB46 and PTGS1 (Fig. 7A). In addition, the high-grade tumor and small-cell NE prostate cancer (SCNC) samples had greater abundances of ZBTB46 and PTGS1 and a lower abundance of SPDEF (Fig. 7B and Supplementary Fig. S6A). Quantitative co-IF staining of the TMA showed that PTGS1 expression was restricted to the NE-like (CHGA-positive) tumor cells (Fig. 7C). Moreover, the ZBTB46 abundance was found to be significantly associated with an upregulated NEPC response signature [14] as confirmed by a GSEA (Fig. 7D). Furthermore, the mean expression correction was validated from the Taylor and TCGA prostate cancer datasets. The results show that ZBTB46 was positively correlated with the NE markers (SYP, CHGA, CHGB, and ENO2) and PTGS1 expression and inversely correlated with the SPDEF and AR-responsive genes (FKBP5, NKX3-1, and KLK3) according to a Pearson coefficient correction analysis (Fig. 7E and Supplementary Fig. S6B). These results indeed support the induction of ZBTB46 and PTGS1 and a reduction in SPDEF as being associated with NEPC differentiation. We extended our analysis to prostate cancer

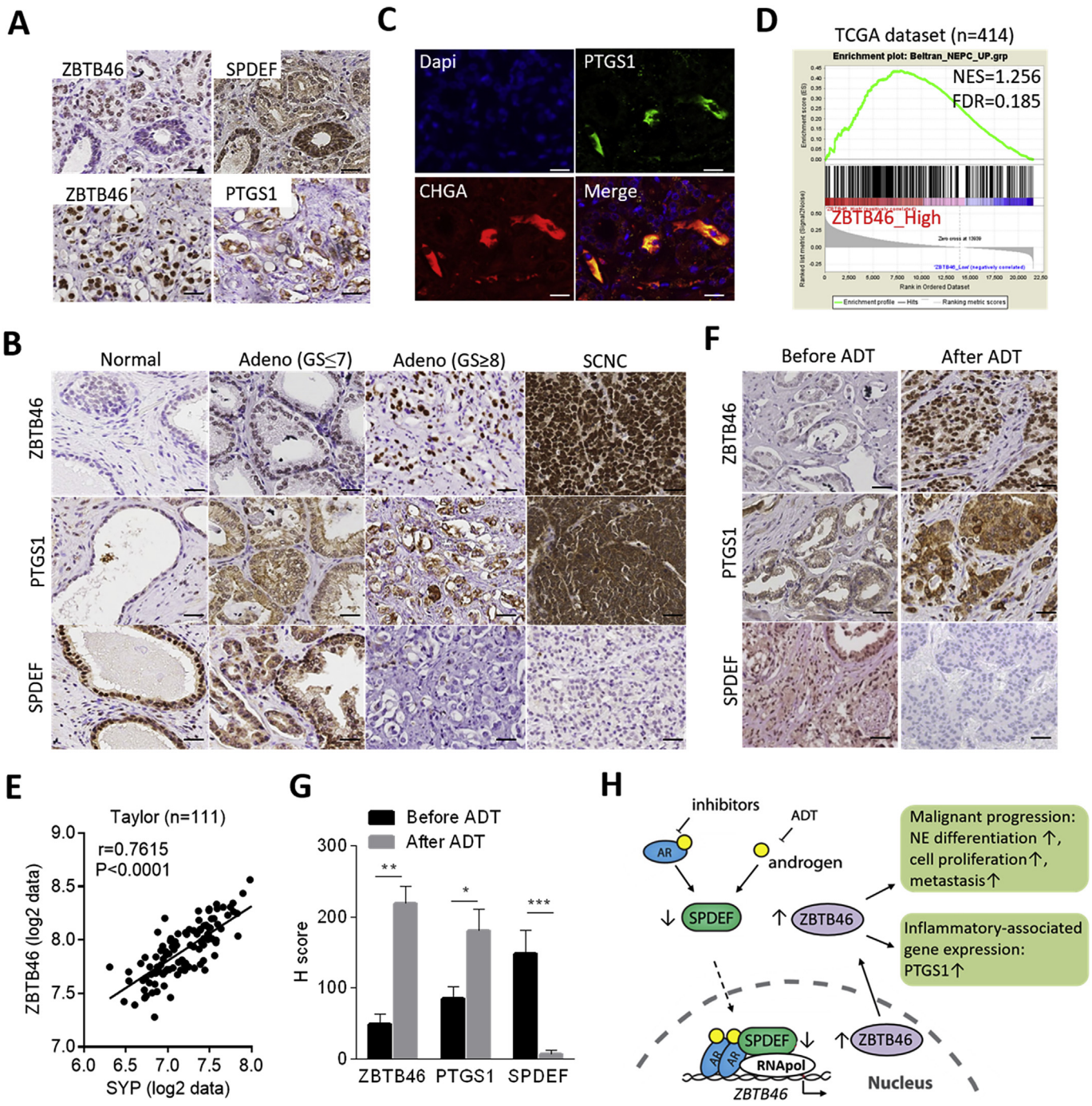

Fig. 7. ZBTB46 is positively correlated with NE markers and PTGS1 in prostate cancer patients. (A) Representative images of IHC staining of consecutive sections of a prostate cancer tissue microarray (TMA) from the Duke University School of Medicine. Scale bars, 100 μm. (B) IHC staining for ZBTB46, PTGS1, and SPDEF in prostate cancer TMA sections containing normal tissues (n = 16), adenocarcinomas with a Gleason score of ≤7 (n = 71), adenocarcinomas with a Gleason score of ≥8 (n = 29), and small-cell NE prostate cancers (SCNCs) (n = 8) from the Duke University School of Medicine. Scale bars, 100 μm. (C) IF staining of the TMA showing coexpression of PTGS1 and CHGA in the same tumor cells. Scale bars, 50 μm. (D) GSEA of TCGA prostate datasets showed that higher ZBTB46 expression of prostate tissues was associated with an NEPC response signaling gene set. (E) Pearson correlation analysis of ZBTB46 with SYP mRNAs in clinical tissue samples from the Taylor prostate cancer dataset. (F and G) IHC staining of ZBTB46, PTGS1 and SPDEF (F) and analysis (G) of nuclear ZBTB46 and SPDEF and cytoplasmic PTGS1 in prostate cancer tissue sections from patients before and after ADT. n = 21 samples from Taipei Medical University-Wan Fang Hospital. Scale bars, 100 μm. Statistical analysis by two-tailed Student's t-test. (H) Proposed model for reducing the activity of AR signaling by ADT or AR inhibitors, which activates ZBTB46, facilitating the development of malignant progression and NE differentiation of prostate cancer cells through the activation of ZBTB46 and PTGS1 by loss of SPDEF.

samples comprising tissue specimens from 21 prostate cancer patients treated before and after ADT collected from Taipei Medical University-Wan Fang Hospital (Taiwan). We found that both nuclear ZBTB46 and cytoplasmic PTGS1 were increased and that nuclear SPDEF was reduced in prostate tumors from the patients who had received ADT compared with the same patients before ADT treatment (Fig. 7F and G). These results suggest that inhibition of AR signaling by ADT may cause a reduction in SPDEF and activation of the ZBTB46-promoting oncogenic signaling pathway components that induce PTGS1 abundance in a subset of patients with aggressive prostate cancer (Fig. 7H).

4. Discussion

In this study, we investigated the gene-regulatory networks that control prostate cancer reprogramming to NEPC and identified novel roles involving the ZBTB46 tumor promoter. We propose a role for ZBTB46 in NEPC progression that is mechanistically linked to the dysregulation of AR signaling pathways through the inactivation of tumor-suppressive SPDEF and activation of PTGS1. We verified that the ZBTB46 oncogenic network potentially regulates PTGS1 expression, is involved in NEPC differentiation, and supports its important pathogenic role in prostate cancer progression. ZBTB46-directed tumorigenesis emerged here as a major determinant of unfavorable outcomes in patients with prostate cancer, and it was associated with the repression of SPDFE. Loss of SPDEF was shown to be involved in metastatic prostate cancer [40]. We demonstrated that SPDEF is the upstream factor that directly regulates ZBTB46. ADT-reduced SPDEF is the mechanism enhancing ZBTB46 upregulation. The aberrant increases in ZBTB46 levels support multiple malignant progressions in prostate cancer, including enhanced cell proliferation, metastasis, and NEPC differentiation competency.

Inhibition of the AR in prostate cancer may contribute the most to malignant transformation and tumors progressing to CRPC [56–58]. We demonstrated a role of the ZBTB46 protein for the NE transformation of prostate cancer and identified ZBTB46 as a novel effector of aberrant AR signaling. A key mechanistic requirement of our findings was the role of ZBTB46 in crosstalk with AR inhibition leading to NEPC differentiation and inflammation-associated gene expression. We demonstrated that ZBTB46 expression is associated with features of NEPC and regulates the expression of PTGS1. We demonstrated that this pathway involves ZBTB46 activation of an antagonist AR program, resulting in NEPC differentiation and the abundance of PTGS1 stimulation in prostate cancer patients with ADT resistance. Our results highlight the role of ZBTB46 in prostate NE transdifferentiation, supporting a model of progressive reprogramming of prostate cancer after ADT.

We elucidated the effect of an anti-inflammatory drug on inducing sensitivity to anti-AR signaling therapy by inhibiting PTGS1. Although we demonstrated that PTGS1 is a direct target of ZBTB46, our results showed that the combination of MDV3100 and a PTGS1 inhibitor can also reduce ZBTB46, suggesting a positive feedback loop between ZBTB46 and PTGS1 or inflammation signaling. An earlier study showed that inflammation signaling is involved in resistance to AR antagonists [30], and we also showed that targeting AR activity induces both ZBTB46 and PTGS1; it is possible that the downstream inflammation signaling of PTGS1 is also involved in the androgen-resistant phenotype, partly through the induction of ZBTB46.

In summary, this study identified a novel molecular determinant of NEPC differentiation, uncovering a broad oncogenic role for the ZBTB46 protein in tumor cell proliferation and malignancy coupled with PTGS1 expression. Our results support a model whereby SPDEF possibly functions as a transcriptional factor to invert the oncogenic role of ZBTB46, promoting NEPC differentiation. In addition, ZBTB46 inhibition may cause inactivation of the ZBTB46-associated inflammatory response proteins, signaling pathway components that affect the biological functions of NEPC cells. Therefore, ZBTB46 is a potential diagnostic marker and therapeutic target in combination with anti-inflammatory drugs in a subset of NEPC patients after ADT.

Authors' contributions

W.Y. Chen and Y.N. Liu designed the experiments and supervised the project. W.Y. Chen, K. C. Jiang, and W.H. Chen performed the experiments. Y.C. Wen, T. Zeng, and J. Huang provided the human prostate cancer samples. W.Y. Chen and Q. Zheng performed the histomorphometric analysis. H.L. Yeh constructed the databases and performed the statistical and bioinformatics analyses. All authors analyzed and interpreted the data. W.Y. Chen, T. Zeng, and Y.N. Liu wrote, reviewed, and/or revised the manuscript.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.004.

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