Enzalutamide, approved by the United States Food and Drug Administration in 2018 for the management of metastatic castration-resistant prostate cancer (CRPC), is an androgen receptor (AR) inhibitor. It blocks androgen binding to the AR, AR nuclear translocation, and AR-mediated DNA binding. Unfortunately, a considerable proportion of tumors eventually develop resistance during the treatment. The molecular mechanisms underlying enzalutamide resistance are not completely understood. Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of polycomb repressor complex 2, has been proposed as a prognostic marker for prostate cancer (PCa). With the goal to test whether EZH2 also plays a critical role in acquisition of enzalutamide resistance in CRPC, here we examined whether EZH2 inhibition/depletion enhances the efficacy of enzalutamide in enzalutamide-resistant PCa cells. We show that combining the EZH2 inhibitor GSK126 with enzalutamide synergistically inhibits cell proliferation and colony formation and promotes apoptosis in enzalutamide-resistant PCa cells. EZH2 depletion also overcomes enzalutamide resistance in both cultured cells and xenograft tumors. Mechanistically, we found that EZH2 directly binds to the promoter of prostate-specific antigen and inhibits its expression in enzalutamide-resistant PCa cells. In agreement, bioinformatics analysis of clinical RNA sequencing data involving GSEA indicated a strong correlation between AR and EZH2 gene expression during PCa progression. Our study provides critical insights into the mechanisms underlying enzalutamide resistance, which may offer new approaches to enhance the efficacy of enzalutamide in CRPC.

Prostate cancer (PCa), the second leading cause of cancer-related death in males in the United States, will have 174,650 new cases and 31,620 deaths estimated in 2019 (1). Because androgen is essential for PCa development, androgen deprivation therapy (ADT) is the mainstay of treatment of advanced PCa. Despite the initial response to ADT, most patients experience disease relapse, and the disease enters a stage called castration-resistant prostate cancer (CRPC) (2). Enough evidence supports the notion that androgen receptor (AR) signaling continues to play a critical role in CRPC (3). Consequently, AR inhibitor enzalutamide was recently approved by the Food and Drug Administration for the treatment of CRPC (4). Unfortunately, development of enzalutamide resistance has already been noted in the majority of patients. Existing resistant mechanisms include de novo androgen biosynthesis, expression of AR splice variants, Wnt/β-catenin pathway activation, and cholesterol biosynthesis (5–8).

Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of the polycomb repressor complex 2 (PRC2), promotes the development and progression of diverse cancers through epigenetic silencing of tumor suppressors by trimethylation of Lys27 on histone H3 (H3K27me3) (9, 10). In addition to its canonical roles in transcriptional silence, several studies have also identified a PRC2-independent function of EZH2 in transcription activation. For example, EZH2 has been implicated in transcriptional activation of NOTCH1, NF-κB target genes, the genes that are regulated by the estrogen receptor, and Wnt signaling transcription factors in breast cancer (11–13). Moreover, EZH2 binds to the β-catenin transcriptional complex and specifically enhances Wnt target genes transactivation in colon cancer independent of its methyltransferase activity (14). EZH2 also functions as a transcriptional coactivator with AR in CRPC (15). Interestingly, the functional switch from a transcriptional silencer to an activator requires S21 phosphorylation of EZH2 by Akt, and activation of AR depends on EZH2 methyltransferase activity. In addition to its known roles in histone modification and transcriptional regulation, EZH2 also methylates a number of nonhistone proteins and modulates their functions.
EZH2 is involved in enzalutamide resistance

For example, EZH2 can methylate STAT3, GATA4, and Jarid2, to regulate their transcriptional activities (16–18). EZH2 can also methylate RORα and PLZF, leading to their ubiquitination and subsequent degradation (19, 20). These findings have highlighted an important role of EZH2 in cancer development and progression. However, the mechanisms governing the oncogenic role of EZH2 in enzalutamide-resistant CRPC remain to be elucidated. In this study, we aim to explore the role of EZH2 in acquisition of drug resistance in PCa. We show here that EZH2 binding to prostate-specific antigen (PSA) promoter suppresses its transcription independent of AR. Accordingly, we show that EZH2 inhibition overcomes enzalutamide resistance, thus enhancing its efficacy in CRPC.

Results

EZH2 and AR were up-regulated in enzalutamide-resistant PCa cells

To probe the potential role of EZH2 in development of chemotherapy resistance in CRPC, we evaluated the possible alteration of EZH2 levels in PCa cells by IB analysis. As indicated, the protein levels of EZH2 were slightly increased, and the levels of AR were clearly elevated in MR49F and C4-2R cells compared with those in LNCaP and C4-2 cells, respectively (Fig. 1A). Fusions of the androgen-regulated gene TMPRSS2 to the oncogenic ETS transcription factor ERG occur in 40–80% of prostate cancers (21). We found that ERG is significantly elevated in enzalutamide-resistant PCa cells compared with that of enzalutamide-sensitive counterparts. Intriguingly, the levels of PSA were dramatically reduced in enzalutamide-resistant cells than those in sensitive cells. To distinguish that the accumulation of EZH2 and AR is due to increased transcription or translation, we also compared mRNA levels of EZH2 and AR in four PCa cell lines (Fig. S1A). We found that mRNA levels of AR were higher in MR49F and C4-2R cells than those in LNCaP and C4-2 cells, consistent with the protein levels. The mRNA levels of EZH2 of MR49F and C4-2R cells were actually lower than those of LNCaP and C4-2 cells, which are consistent with our RNA-seq results (Fig. S1, B and C), indicating that additional post-translation mechanisms likely contribute to EZH2 elevation during acquisition of enzalutamide resistance.

Inhibition of EZH2 restores sensitivity toward enzalutamide in enzalutamide-resistant cells

To further explore the effects of EZH2 on acquisition of enzalutamide resistance, we treated enzalutamide-resistant cells with EZH2 inhibitor GSK126, an SAM competitive binder of EZH2 that inhibits its histone methyltransferase activity and induces a loss of H3K27 trimethylation without affecting the levels of total histone H3 or other PRC2 components (22). We investigated whether GSK126 and enzalutamide act synergistically to inhibit the growth of enzalutamide-resistant cells. First, C4-2R, MR49F, and 22RV1 cells were treated with GSK126 and/or enzalutamide and subjected to IB to follow cell death and proliferation. Increased levels of apoptotic markers (cleaved PARP and cleaved caspase 3) and reduced levels of proliferation marker (proliferating cell nuclear antigen) indicated that combination treatment of GSK126 and enzalutamide led to a significantly increased cellular apoptosis compared with GSK126 or enzalutamide treatment alone (Fig. 1B). Next, combination treatment of GSK126 and enzalutamide showed a much stronger inhibitory effect on both cell proliferation and colony formation in C4-2R, MR49F, and 22RV1 cells (Fig. 1, C–F). To further confirm the synergistic effect of two drugs, cell apoptosis was analyzed by flow cytometry. There was no apparent apoptosis induced by enzalutamide treatment alone and a mild increase in early apoptosis induced by GSK126 treatment alone. However, we observed significantly increased populations of late apoptosis upon combination treatment compared with the mono treatment (Fig. 1G). Moreover, we used the Chou–Talalay method to examine the combination index of GSK126 and enzalutamide as well. The combination index values are 0.744 and 0.85 in C4-2R and 22RV1, respectively, which means significant synergic effects when both drugs are administered together (Fig. S1D).

To address potential off-target effect associated with pharmacologic inhibition of EZH2, we depleted EZH2 with lentivirus-encoded shRNA in C4-2R and 22RV1 cells (Fig. 2A). Knockdown of EZH2 suppressed the growth of these cells, which was more significant following combinatory treatment with enzalutamide (Fig. 2B). Combinatory treatment with enzalutamide also significantly inhibited colony formation in these two cell lines (Fig. 2C). To further confirm the findings of synergic effects of combinatory treatment on cell apoptosis, we treated cells (depleted with or without EZH2) with enzalutamide, followed by IB against cleaved PARP. As shown in Fig. 2D, enzalutamide treatment dramatically increased apoptosis in cells with EZH2 depletion. In a further step, we then treated the EZH2 knockdown cells with GSK126 to determine whether there are further effects on death and proliferation, in the presence and absence of enzalutamide. As shown in Fig. S1E, GSK126 treatment did not induce more cleaved PARP and proliferating cell nuclear antigen in the presence and absence of enzalutamide, which indicates there are no further effects of GSK126 on EZH2 knockdown cells. Altogether, these results demonstrate that EZH2 inhibition can re sensitize the resistant cells toward enzalutamide, suggesting a strong synergistic effect between GSK126 and enzalutamide.

Target depletion of EZH2 enhances the efficacy of enzalutamide in enzalutamide-resistant xenograft tumors

To assess this combinatory effect in vivo, we next tested the effect of combination treatment in a 22RV1-derived xenograft mouse model. To rule out the potential off-target effect of GSK126 on tumor suppression in vivo, a more stable genetic approach (EZH2 shRNA) was used. As shown in Fig. 3 (A and B), EZH2 depletion slightly decreased tumor volumes, whereas administration of enzalutamide showed synergistic effects, indicating that knockdown of EZH2 overcame enzalutamide resistance and restored sensitivity to enzalutamide. Although both wet weights and sizes of the tumors were reduced with EZH2 knockdown alone or monotherapy of enzalutamide, the effect was much more significant with EZH2 depletion plus enzalutamide treatment (Fig. 3, C and D). It is important to point out that the combination treatment did not affect animal weights (Fig. 3E). Histologically, H&E staining of tumors from
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**A**

| LNCaP | MR49F | C4-2 | C4-2R |
|-------|-------|------|-------|
| EZH2  | +     | -    | -     |
| AR    | +     | +    | +     |
| PSA   | -     | -    | -     |
| ERα   | -     | -    | -     |
| GAPDH | -     | -    | -     |

**B**

| C4-2R (72h) |
|-------------|
| GS1K26 (20μM) | ENZA (30μM) |
| -           | +           |
| +           | -           |

**C**

- **C4-2R**
  - Cell viability (%)
  - Time (Days)
- **MR49F**
  - Cell viability (%)
  - Time (Days)
- **22RV1**
  - Cell viability (%)
  - Time (Days)

**D**

| C4-2R | MR49F | 22RV1 |
|-------|-------|-------|
| DMSO  | GSK126 | ENZA  |
| ENZA  | COMBO  |

**E**

- **C4-2R**
  - MR49F
  - 22RV1

**F**

- **C4-2R**
  - Num of Clones
  - DMSO vs. GSK126
  - GSK126 vs. ENZA
  - ENZA vs. COMBO

**G**

- **C4-2R**
  - Annexin V
  - PI

- **MR49F**
  - Annexin V
  - PI

- **22RV1**
  - Annexin V
  - PI
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sh-Ctl without treatment showed numerous mitotic cells, suggesting that cell proliferation was active (Fig. 3F). However, tumors derived from EZH2 knockdown cells plus treatment with enzalutamide showed increased numbers of apoptotic bodies with condensed cytoplasm and pyknotic nuclei compared with other groups. To determine whether EZH2 knockdown alone or in combination with enzalutamide represses tumor proliferation and promotes apoptosis, tumor slices were analyzed by immunofluorescence staining for Ki67 and cleaved caspase 3 (Fig. 3, G and H). A significant increase in cleaved caspase 3-positive cells, along with a reduction in Ki67-positive cells, was observed after combination treatment, indicating a strong induction of cell apoptosis and inhibition of cell proliferation. To further validate that EZH2 depletion sensitizes the tumors to enzalutamide, we conducted another study with xenograft tumors derived from C4-2R cells. Consistent with previous findings, EZH2 depletion plus enzalutamide inhibited the tumor growth significantly (Fig. S2, A–E). A remarkable increase in the number of apoptotic bodies was also observed upon treatment with enzalutamide (Fig. S2F). Immunostaining for Ki67 and cleaved caspase 3 also confirmed that the tumors after enzalutamide therapy had a significant reduction in overall proliferation and a significant increase in apoptosis (Fig. S2, G–J). In summary, these results, consistent with cell-based studies, suggest that GSK126 and enzalutamide can act synergistically both in vitro and in vivo, providing a novel and promising therapeutic option to treat CRPC patients.

EZH2 represses AR signaling in enzalutamide-resistant cells

To investigate the mechanism underlying the observed synergistic effect, C4-2R, MR49F, and 22RV1 cells were treated with GSK126, enzalutamide, or GSK126 plus enzalutamide, followed by IB. As indicated, the protein levels of EZH2, AR, PSA, and ERG were all remarkably reduced with combination treatment compared with monotherapy (Fig. 4A). This result suggests that both EZH2 and AR signal pathway are crucial to the proliferation of enzalutamide-resistant cells. Then we asked whether AR signaling pathway is affected by EZH2 in enzalutamide-resistant cells. As described above, the protein levels of both EZH2 and AR were elevated in MR49F and C4-2R compared with those in their enzalutamide-sensitive parental cells (Fig. 1A). However, we always detected much lower levels of PSA protein in enzalutamide-resistant cells, suggesting that AR pathway is largely inactive in enzalutamide-resistant cells (Fig. 1A). Next, we attempted to investigate the mechanisms by which EZH2 affects AR signaling in enzalutamide-resistant cells. A recent study reported that EZH2 as a transcriptional activator can directly induces AR gene expression in a polycomb- and methylation-independent manner (23). To investigate whether EZH2 directly occupies the AR promoter region, EZH2 ChIP assays were performed in C4-2R and MR49F cells. Apparent EZH2 occupancy at AR promoter was observed in both cell lines (Fig. 4B). Moreover, ChIP-qPCR assays were performed on reported AR target genes, such as PSA and TMPRSS2. EZH2 occupancy on PSA and TMPRSS2 promoters was observed as well (Fig. 4B). To further investigate the roles of EZH2 on AR, PSA and TMPRSS2 transcription, H3K27me3 ChIP-qPCR assays were performed. Again, occupancy of H3K27me3 at AR, PSA, and TMPRSS2 promoter regions were detected (Fig. 4C). To further demonstrate that EZH2 regulates AR, PSA, and TMPRSS2 transcription, GSK126 was used to treat enzalutamide-resistant cells with increasing doses (5, 10, and 15 μM). As indicated, quantitative RT-PCR (qRT-PCR) analysis demonstrated that the AR mRNA levels slightly increased after treatment. Interestingly, the levels of PSA and TMPRSS2 mRNA increased significantly upon GSK126 treatment (Fig. 4D). Meanwhile, GSK126 treatment increased the expression of another AR-target gene FKBP5 in MR49F and 22RV1 cells. As positive controls, GSK126 treatment restored expression of reported EZH2 target genes, such as SLIT2 and CNR1. Interestingly, EZH2 mRNA expression was up-regulated to some extent upon GSK126 treatment. These results suggest that EZH2 largely remains as an epigenetic silencer on AR signaling pathway in enzalutamide-resistant cells. Furthermore, IB analysis demonstrated that PSA protein levels increased slightly upon GSK126 treatment, whereas the levels of AR and ERG slightly decreased (Fig. 4A). In agreement, knockdown of EZH2 in C4-2R and 22RV1 cells increased the levels of PSA and decreased the levels of AR and ERG (Fig. 4E). To further validate that EZH2 suppresses the transcription of PSA, we performed reporter gene assays with PSA gene enhancer and promoter-linked luciferase. As indicated, overexpression of EZH2 in 293T cells led to reduced luciferase activity, suggesting that EZH2 indeed down-regulates the PSA expression (Fig. 4F). Moreover, PSA depletion alone can suppress proliferation of MR49F and C4-2R cells, which can be enhanced by GSK126 treatment (Fig. 4, G and H). A previous study has demonstrated that EZH2 directly interacts with AR to modulate AR function (24). Therefore, we then examined the role of EZH2/AR complex in regulating enzalutamide-resistance in CRPC. In agreement, EZH2 directly interacts with AR in both sensitive and resistant cells. Intriguingly, stronger interaction was found in enzalutamide-resistant cells than that in enzalutamide-sensitive cells. These results indicate that the EZH2–AR complex at least partially contributes to regulation of enzalutamide resistance in CRPC (Fig. S2K). This result indi-
cates that PSA pathway still works in enzalutamide-resistant PCa. In support, EZH2 inhibition elevated the level of PSA, resulting in a synergic effect of inhibition of EZH2 and PSA depletion. In conclusion, our data support the notion that EZH2 plays an epigenetic silence role on PSA expression in enzalutamide-resistant PCa cells.

Correlation of AR and EZH2 expression during PCa progression

To determine the clinical relevance of our findings, we analyzed expression levels of AR and EZH2 in 497 tumors from TCGA database. As shown in Fig. 5 (A and B) expression levels of both EZH2 and AR were increased in tumors compared with
EZH2 is involved in enzalutamide resistance

A

DAYS POST TREATMENT

sh-CtI  
sh-EZH2  
sh-CtI+ENZA  
sh-EZH2+ENZA

B

sh-CtI  
sh-EZH2  
sh-CtI + ENZA  
sh-EZH2 + ENZA

C

Tumor Weight (g)

sh-CtI  
sh-EZH2  
sh-CtI+ENZA  
sh-EZH2+ENZA

D

Average Tumor Size (mm)

sh-CtI  
sh-EZH2  
sh-CtI+ENZA  
sh-EZH2+ENZA

E

Body Weight (g)

sh-CtI  
sh-EZH2  
sh-CtI + ENZA  
sh-EZH2 + ENZA

F

H&E

50 µm  50 µm  50 µm  50 µm

sh-CtI  
sh-EZH2  
sh-CtI + ENZA  
sh-EZH2 + ENZA

G

DAPI/Ki67

50 µm  50 µm  50 µm  50 µm

sh-CtI  
sh-EZH2  
sh-CtI + ENZA  
sh-EZH2 + ENZA

DAPI/Cleaved Caspase 3

50 µm  50 µm  50 µm  50 µm

sh-CtI  
sh-EZH2  
sh-CtI + ENZA  
sh-EZH2 + ENZA

H

Ki67

Percentage of positive staining (%)

sh-CtI  
sh-EZH2  
sh-CtI+ENZA  
sh-EZH2+ENZA

Cleaved Caspase 3

Percentage of positive staining (%)

sh-CtI  
sh-EZH2  
sh-CtI+ENZA  
sh-EZH2+ENZA
adjacent normal tissues. Next, we further explored the correlation between the levels of EZH2 and AR and observed that there was a strong correlation between the levels of these two genes in tumors \((r = 0.5675\) but not of those in normal tissues \((r = 0.1806)\) \((\text{Fig. S1})\). Next, we compared expression levels of EZH2 and AR in enzalutamide-sensitive versus -resistant cell lines. As indicated in \(\text{Fig. S1B}\), the levels of AR were dramatically increased in enzalutamide-resistant cells compared with enzalutamide-sensitive cells \((C4-2\text{ versus }C4-2R\) and LNCaP versus MR49F\). We did not observe significant changes for transcription levels of EZH2 in cells with different sensitivities to enzalutamide \((\text{Fig. S1C})\). To further investigate potential correlations among EZH2, AR, and ADT, we analyzed expression levels of these two genes in seven pairs of PCa patients pre- and post-ADT \((\text{GSE48403})\). As shown in \(\text{Fig. 5 (D and E)}\), we found that expression levels of EZH2, but not AR, were significantly suppressed upon ADT. Finally, AR expression was significantly elevated in high risk PCa patients \((\text{Gleason score } = 10)\). Consistently, EZH2 expression was also gradually increased with disease progression compared with normal tissues \((\text{Fig. 5, F and G})\). These data indicate that EZH2 expression correlates with that of AR, and such a correlation is associated with disease progression.

**Bioinformatics analysis revealed that metastasis contributes to enzalutamide-resistance**

To explore signaling pathway(s) involved in the disease progression associated with EZH2 and AR, gene set enrichment analysis \((\text{GSEA})\) was performed. As indicated, metastasis pathway was found to be activated upon up-regulation of both EZH2 \((\text{rank } 3)\) and AR \((\text{rank } 22)\) \((\text{Fig. 6, A and B, and Fig. S1, F and G})\). To further determine genes that contribute to enzalutamide resistance, we then overlapped RNA-seq data from both cell lines, including enzalutamide-sensitive and -resistant cells, and human patient tumors. As illustrated in \(\text{Fig. 6 (C and D)}\), 38 metastasis-related genes were found to correlate with increase of both AR and EZH2 expression \((\text{Table S2})\). To further validate our findings, we then performed IB against markers for epithelial-to-mesenchymal transition \((\text{EMT})\). We found that the levels of E-cadherin were decreased in enzalutamide-resistant cells compared with those in enzalutamide-sensitive cells. At the same time, the levels of N-cadherin and vimentin were dramatically increased in MR49F and C4-2R cells compared with LNCaP and C4-2 cells \((\text{Fig. 6E})\). Meanwhile, Snail, an important transcription factor driving EMT, was also dramatically up-regulated in enzalutamide-resistant cells compared with their enzalutamide-sensitive counterparts. These data suggest that expression levels of both AR and EZH2 correlate with metastasis, which contributes to enzalutamide resistance in PCa.

**Discussion**

EZH2 was first reported to be elevated in hormone-refractory, metastatic PCa. Although its depletion inhibits cell proliferation, overexpression of EZH2 leads to transcription repression of a specific set of genes in prostate cells. Furthermore, a high expression of EZH2 correlates to a poor prognosis of PCa. Based on these findings, it was concluded that EZH2 might be a good diagnostic marker to distinguish indolent PCa from those at high risk of lethal progression \((\text{25})\). Interestingly, it was later shown that EZH2 oncogenic activity in CRPC cells is polycomb-independent. EZH2 actually acts as a transcription coactivator for critical transcription factors such as AR, and this functional switch is driven by AKT-dependent phosphorylation at Ser 21 \((\text{15})\). The latter finding was further confirmed recently by showing that EZH2 activates AR via its direct occupancy at the AR promoter. Although depletion of EZH2 down-regulates AR, its overexpression increases AR mRNA and protein. Further, EZH2 activates AR independently of its histone methyltransferase activity \((\text{23})\). In a further step, we aimed to determine whether EZH2 also plays a critical role in acquisition of enzalutamide-resistance in CRPC cells. We showed that a combination of GSK126 and enzalutamide inhibits proliferation and colony formation of enzalutamide-resistant CRPC cells in a synergistic manner. Further, shRNA-mediated depletion of EZH2 clearly enhanced the efficacy of enzalutamide in enzalutamide-resistant CRPC cells and xenograft tumors that was consistent with established finding \((\text{26})\). Mechanistically, we showed that EZH2 directly loads onto the promoters of AR, PSA, and TMPRSS2. Although GSK126 treatment did not seem to dramatically affect the mRNA levels of AR, it did significantly increase the mRNA levels of PSA and TMPRSS2 in both C4-2R and MR49F cells, suggesting that EZH2 acts as a negative regulator of PSA and TMPRSS2 in enzalutamide-resistant cells. To support this, depletion of EZH2 with shRNA clearly increased the levels of PSA protein but showed mild reduction of the levels of AR and ERG in C4-2R and 22RV1 cells. Therefore, EZH2-associated inhibition of PSA and TMPRSS2 could be AR-independent in enzalutamide-resistant CRPC cells. It was known that ~50% PCas have chromosomal fusions between the androgen-regulated gene TMPRSS2 to the oncogenic ETS transcription factor ERG and that ERG disrupts AR signaling by inhibiting AR signaling. Further, ERG-associated inhibition of AR is EZH2-dependent \((\text{10})\). Because ERG is significantly elevated in enzalutamide-resistant PCa cells compared with their enzalutamide-sensitive counterparts, this could be another reason to explain the reduced PSA levels in enzalutamide-resistant cells. Because therapy resistance is usually associated with...
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metastasis, it is not surprising that our human patient data-based bioinformatics analysis showed an enrichment of metastasis pathway shared by both activation of AR and EZH2.

Various mechanisms have been described to be responsible for acquisition of enzalutamide resistance in CRPC, including AR mutations, expression of AR variants, activation of the Wnt/β-catenin pathway (8), activation of polo-like kinase 1 (27), and elevation of cholesterol biosynthesis (7). Identification of EZH2 as an additional factor whose inhibition overcomes enzalutamide resistance as described here provides another option for physicians to use to enhance the efficacy of enzalutamide in clinics. The data presented support an immediate clinical trial to combine various existing EZH2 inhibitors with enzalutamide for CRPC patients.

**Experimental procedures**

**Cell culture and drugs**

LNCaP, MR49F, C4-2, C4-2R, and 22RV1 cells were used in the study. Although growth of LNCaP cells are androgen-dependent, C4-2 cells were derived from LNCaP but are androgen-independent. MR49F cells were derived from LNCaP cells but are enzalutamide-resistant. C4-2R cells were derived from C4-2 but are enzalutamide-resistant. LNCaP and 22RV1 cells were purchased from ATCC. C4-2 cells were obtained from the M. Anderson Cancer Center. MR49F and C4-2R cells were kindly provided by Dr. Amina Zoubeidi at the Vancouver Prostate Cancer Center and Dr. Allen Gao at University of California at Davis, respectively. LNCaP, C4-2, and 22RV1 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C in a humidified incubator with 5% carbon dioxide. C4-2R and MR49F cells were cultured in previously described medium containing 20 and 10 μM enzalutamide, respectively. Enzalutamide and GSK126 were purchased from Selleckchem. Puromycin and MTT was purchased from Sigma.

**IB analysis and antibodies**

Harvested cells were lysed in TBSN buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1.5 mM EDTA, 5 mM EGTA, 0.5% Nonidet P-40, and 0.5 mM Na3VO4) supplemented with protease inhibitors and phosphatase inhibitors. Upon lystate collection, protein concentrations were measured using Pierce BCA protein assay kit (Thermo, catalog no. 23225). Equal amounts of proteins from each sample were loaded and resolved by SDS-PAGE. Upon transferring to polyvinylidene difluoride membranes, the proteins were probed with indicated antibodies. Antibodies against H3K27me3 and cleaved PARP were purchased from Active Motif and Millipore, respectively. All other antibodies were obtained from Cell Signal Technology.

**Cell viability assay**

The cells were seeded in 96-well plates, cultured for 24 h, and treated with different concentrations of the drugs, followed by incubation with MTT for 4 h on the indicated days. Upon resolving the crystal with 100 μl of DMSO, the cells were subjected to measurement of the absorbance at 570 nm on a plate reader.

**Colony formation assay**

Cells (2,000–5,000/well) were seeded in 6-well plates and cultured in medium alone or containing different drugs for 14 days, with medium refreshment every 7 days. After culturing, the cells were fixed in 10% formalin and stained with 0.5% crystal violet for 30 min, followed by counting of colony numbers.

**Flow cytometry analysis**

The cells were cultured in medium alone or containing different drugs, trypsinized without EDTA, doubly stained with annexin V and polyvinylidene difluoride (PI) using a Pacific blue annexin V apoptosis kit (Biolegend, catalog no. 640928) according to the manufacturer’s instruction and analyzed by flow cytometry (BD LSRRFORTESSA). The data were analyzed with FlowJo software.

**qRT-PCR**

After total RNA was extracted from cells using the RNeasy mini kit (Qiagen, catalog no. 74104) according to the manufacturer’s instruction, complementary DNA was synthesized using SuperScript IV VILO Master Mix (Thermo, catalog no. 11766500) from same amount of RNA. qRT-PCR were performed using FastStart Universal SYBR Green Master (Roche, catalog no. 04913850001) and Roche LightCycler 96 thermocycler (Roche Diagnostics Corp.). The relative quantification of gene expression for each sample was analyzed by the threshold cycle (CT) method. All individual reactions were performed in triplicate, and all genes were normalized to glyceraldehyde-3-phosphate dehydrogenase. Information of primers is provided in Table S1.

**ChIP assay**

ChIP of EZH2 was performed using EZ-Magna ChIP A/G kit (Millipore, catalog no. 17-10086) according to the manufacturer’s protocol. In brief, the cells were cross-linked with 1% formaldehyde for 10 min at room temperature and then quenched with 0.125 mM glycine. After washing, the cells were sonicated on Bioruptor ultrasonicator (Diagenode). ChIP enrichment was assessed using quantitative PCR with primers indicated in Table S1.

Figure 4. EZH2 represses AR signaling pathway in enzalutamide-resistant cells. A, cells were treated with GSK126, enzalutamide, or both and harvested for IB with indicated antibodies. B, ChIP-qPCR shows EZH2 binding to promoter regions of AR and its target genes in MR49F and C4-2R cells. C, ChIP-qPCR shows H3K27me3 binding to promoter regions of AR and its target genes in MR49F and C4-2R cells. D, C4-2R, MR49F, and 22RV1 cells were treated with GSK126 (72 h for C4-2R, 48 h for MR49F and 22RV1) and subjected to ChIP-qPCR analysis. E, C4-2R and 22RV1 cells were infected with control shRNA or shEZH2 lentivirus, followed by IB analysis. F, EZH2 overexpression represses PSA. 293T cells were cotransfected with EZH2 plasmid and a PSA promoter reporter construct along with pRL-TK (the internal control), incubated for 48 h, and then monitored for luciferase activity. G, C4-2R and MR49F cells were infected with control shRNA or sh-PSA lentivirus, followed by IB analysis. H, C4-2R and MR49F cells were depleted of PSA with shRNA, then treated with GSK126 (8 μM), and harvested for MTT assays. I, proposed working model based on the results of this study. The data shown are means ± S.D. of technical replicates from one representative experiment of three.*, 0.01 < p < 0.05; **, 0.001 < p < 0.01; ***, p < 0.001. ENZA, enzalutamide; Ctrl, control.
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The cells were infected with lentivirus expressing shRNA to deplete EZH2 or PSA (Sigma). Upon 2 weeks’ selection with puromycin, a pool of cells with EZH2 or PSA knockdown was obtained. The PSA luciferase reporter (PSA-Luc) that contains ~5.8-kb genomic fragment from the promoter of the PSA gene and Renilla luciferase reporter vector were obtained from Prof. Haojie Huang. For luciferase reporter assays, the cells were

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**Figure 5. Correlation of AR and EZH2 expression during PCa progression.** A and B, gene expression of AR (A) and EZH2 (B) in PCa tumors (497 cases) and adjacent normal tissues (52 cases). C, correlation between AR and EZH2 gene expression in PCa and adjacent normal tissue. D and E, gene expression changes of AR (D) and EZH2 (E) upon ADT treatment. F and G, changes of AR (F) and EZH2 (G) expression during PC progression.

**Cell transfection and luciferase reporter assay**

The cells were infected with lentivirus expressing shRNA to deplete EZH2 or PSA (Sigma). Upon 2 weeks’ selection with puromycin, a pool of cells with EZH2 or PSA knockdown was obtained. The PSA luciferase reporter (PSA-Luc) that contains ~5.8-kb genomic fragment from the promoter of the PSA gene and Renilla luciferase reporter vector were obtained from Prof. Haojie Huang. For luciferase reporter assays, the cells were
Figure 6. Expression of metastasis-related genes correlates to both AR and EZH2. A and B, metastasis signal pathway was activated in AR and EZH2 high tumors revealed by GSEA. C, heat map of metastasis-related genes whose expression correlates to AR and EZH2. D, expression levels of 38 metastasis-related genes were commonly associated with AR and EZH2. E, IB analysis of EMT markers in enzalutamide-sensitive versus -resistant PCa cells.
transfected with plasmids for PSA-Luc firefly and Renilla luciferase reporter genes using Lipofectamine 2000 reagent (Invitrogen). After 48 h of culture, the cells were harvested, and firefly and Renilla luciferase activities in cell lysates were measured using a dual luciferase kit (Promega, catalog no. E1910). Renilla luciferase activities of cells were used as internal control. Relative luciferase units were determined by normalizing the firefly units with the Renilla activity.

**Xenograft experiments**

For xenograft, the cells (2 x 10^6 cells/mouse) were suspended in 200 μl of culture medium with 50% Matrigel matrix (Corning, catalog no. 354234) and injected subcutaneously into the dorsal flank of the nude mice (Harlan Laboratories) 1 week after surgical castration. Tumor-bearing mice were randomly divided into four groups and treated with 200 μl of vehicle control or enzalutamide (20 mg/kg) by oral gavage. Tumor volumes were measured with digital caliper every 4 days and calculated with the formula, \( V = L \times W \times H \) (where \( V \) is volume, \( L \) is length, \( W \) is width, and \( H \) is height). For histology and immunofluorescent analysis, xenograft tumors were fixed in 10% formalin and embedded in paraffin, sectioned to 5 μm, and stained using conventional H&E staining. Immunofluorescent chemistry staining was accomplished with the M.O.M kit (Vector Laboratories). The mouse experiments were approved by the Purdue Animal Care and Use Committee.

**Statistical analysis**

The statistical significance of the results was analyzed using an unpaired Student’s t test (GraphPad software). A p value of less than 0.05 indicates statistical significance.

**Bioinformatics analysis**

RNA-seq data and their associated clinical information of 497 primary PCa and 52 adjacent normal tissues were obtained from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/). RNA-seq data of seven pairs of patients before and after treatment of ADT for ~22 weeks was collected from Gene Expression Omnibus, GSE48403. Our in-house data include RNA-seq results of enzalutamide-sensitive (LNCaP and C4-2) and enzalutamide-resistant (MR49F and C4-2R) cells. GSEA with MSigDB V6.2 C5 Gene Ontology gene sets was used to identify the relevant pathways enrichment by comparing gene expression profiles in benign hyperplasia versus untreated primary PCa, AR-high versus AR-low in PCa, and EZH2-high versus EZH2-low in PCa.

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