NOTCH signaling is activated in and contributes to resistance in enzalutamide-resistant prostate cancer cells

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Abstract

Prostate cancer is the second leading cause of cancer death among men in the United States. The androgen receptor (AR) antagonist enzalutamide is an FDA-approved drug for treatment of patients with late-stage prostate cancer and is currently under clinical study for early-stage prostate cancer treatment. After a short positive response period, tumors will develop drug resistance. In this study using RNA-Seq and bioinformatics analyses, we observed that NOTCH signaling is a deregulated pathway in enzalutamide-resistant cells. NOTCH2 and c-MYC gene expression positively correlated with AR expression in samples from patient with hormone refractory disease in which AR expression levels correspond to those typically observed in enzalutamide-resistance. Cleaved-NOTCH1, Hes family BHLH transcription factor 1 (HES1) and c-MYC protein expression levels are elevated in two enzalutamide-resistant cell lines, MR49F and C4-2R indicating NOTCH signaling activation. Moreover, inhibition of the overexpressed ADAM metallopeptidase domain 10 (ADAM10) in the resistant cells induces an exclusive reduction in cleaved-NOTCH1 expression. Furthermore, exposure of enzalutamide-resistant cells to both PF-03084014 and enzalutamide increased cell death, decreased colony formation ability, and re-sensitized cells to enzalutamide. Knockdown of NOTCH1 in C4-2R increased enzalutamide sensitivity by decreasing cell proliferation and increasing cleaved-PARP expression. In a 22RV1 xenograft model, PF-03084014 and enzalutamide decreased tumor growth through reducing cell proliferation and increasing apoptosis. These results indicate that NOTCH1 signaling may contribute to enzalutamide-resistance in prostate cancer and inhibition of NOTCH signaling can re-sensitize resistant cells to enzalutamide.
**Introduction**

Prostate cancer is the most diagnosed cancer in men in the United States (1). Despite the high survival rate of prostate cancer patients, most develop resistance to therapies leading to a more aggressive form of the disease and ultimately death. Enzalutamide, initially approved as a second-line treatment of metastatic prostate cancer patients, has shown significant beneficial results when used as first-line therapy for patients with metastatic prostate cancer or even early stage prostate cancer in combination with hormonal therapy (2, 3). Shortly after a brief response period to enzalutamide patients will develop resistance leading to death (4). With enzalutamide in ongoing clinical trial on early stage prostate cancer patients, it is of high importance to understand the mechanism by which prostate cancer patients develop resistance to enzalutamide and identify novel approaches to overcome resistance. By understanding and identifying these mechanisms, targeted combination therapies can be used to treat patients who develop drug resistance.

In general, androgen deprivation therapy (ADT)-resistance and specifically enzalutamide-resistance mechanisms mainly highlight the role of the Androgen Receptor (AR) in resistance development, including AR amplification, activating mutations, and AR variants (5, 6). In this study, we hypothesized that genes and pathways outside of the AR signaling pathway contribute to enzalutamide-resistance in prostate cancer cells. To test our hypothesis, we used RNA-sequencing to compare previously developed enzalutamide-resistant cell lines to their parental cell lines to identify potential non-AR pathways that contribute to resistance. Our results show that the Notch signaling pathway is linked to enzalutamide-resistance. Abrogation of this pathway in vitro and in vivo restores sensitivity of prostate cancer cells to enzalutamide.

**Results**

*Long-term enzalutamide treatment of prostate cancer cell lines induces global gene expression changes after acquiring resistance.* Kuruma et al. and Liu et al. developed enzalutamide-resistant cell lines MR49F and C4-2R derived from the parental cell lines LNCaP and C4-2 respectively (5, 6). These cell lines were developed by extensive treatment with high concentrations of enzalutamide in a tissue culture setting or in an in vivo xenograft mouse model to serve as a model to study enzalutamide-resistance (5, 6). To compare enzalutamide-sensitive to enzalutamide-resistant cell lines, RNA was isolated from LNCaP, C4-2, MR49F and C4-2R after a 4-hour treatment with enzalutamide. Around 30000 features were detected in all cell lines after RNA-sequencing, excluding genes with zero counts. Sample-to-sample matrix heatmap reveals that the biological replicates are consistent within each sample group (Fig. 1A). In addition, principle component analysis (PCA) shows that our replicates cluster together within each group (Fig. 1B). Figures 1A and 1B show that LNCaP is the closest to MR49F. On the other hand, C4-2 has the most similarity with C4-2R. This validates our choice for pairing LNCaP with MR49F and C4-2 with C4-2R. For differentially expressed genes (DEG) analysis we used 3 different algorithms, DESeq2, EdgeR and Cufflinks. Using EdgeR with a 1% false discovery rate and 2-fold change cutoff we were able to identify a significant number of genes that are differentially expressed in LNCaP vs MR49F and C4-2 vs C4-2R (Fig. 1C). Overall, the intersection of the 3 algorithms showed 901 genes that have been differentially expressed in MR49F compared to LNCaP and 1266 genes differentially expressed in C4-2R compared to C4-2 (Fig. 1D). Finally, Table 1 and Table 2 show the number of genes that are up- or down-regulated in LNCaP vs MR49F and C4-2 vs C4-2R discovered by the independent algorithms. Our data indicates that long-term enzalutamide treatment leads to alteration of global gene expression.

Notch signaling pathway is enriched in patient samples that mimic enzalutamide-resistant cell lines. Our bioinformatics data from comparing enzalutamide-resistant to enzalutamide-sensitive cell lines revealed that the normalized counts of AR were significantly and consistently increased in
MR49F and C4-2R compared to LNCaP and C4-2 respectively (Fig. 2A). This indicates that cell lines with acquired resistance exhibit a higher expression of AR compared to cells that are still sensitive the drug. Due to the lack of clinical samples from patients treated with enzalutamide, we analyzed a cohort of 72 patients with hormone-refractory prostate cancer (Fig. 2B). Of the 72 patients, 26 had a high AR gene expression mimicking cell lines that are resistant to enzalutamide and 46 had a low AR gene expression mimicking cell lines that are sensitive. After separating the samples into AR low vs. AR high, the dataset was subjected to gene set enrichment analysis (GSEA). Genes from the “pre-Notch transcription and translation signaling” and “pre-Notch expression and processing signaling” pathways are significantly enriched in the AR high group (Fig. 2C and D). These pathways are subgroups of the Notch signaling pathway from Reactome. Furthermore, we used Pearson correlation analysis to identify genes that correlate with AR expression in the dataset. NOTCH2 expression positively correlated with AR expression, with a correlation coefficient of 0.4331 (Fig. 2E and G). In addition, one of the downstream targets of Notch signaling c-MYC positively correlated with AR expression, with a correlation coefficient of 0.4536 (Fig. 2F and H). These data indicate that Notch signaling pathway may play a role in resistance to enzalutamide in prostate cancer.

Expression of Notch signaling pathway genes is deregulated in enzalutamide-resistant cells. To further explore the implication of Notch signaling in resistance, we compared the expression of genes from this pathway in enzalutamide-resistant vs. enzalutamide-sensitive cell lines. As shown in Figures 3A and 3B, more than 30 genes from the Notch signaling pathway are either up- or down-regulated in MR49F and C4-2R compared to LNCaP and C4-2 respectively (Table S1).

To investigate the functional features of the Notch signaling pathway in enzalutamide-resistant cell lines we examined the protein expression of important genes from this pathway. Notch family of proteins is composed of 4 transmembrane receptors (NOTCH 1, 2, 3, and 4) that are cleaved upon ligand-binding on the cell surface rendering their activation and leading to downstream events (7–9). Our results show that cleaved-NOTCH1 has a higher expression in MR49F and C4-2R compared to their sensitive counterparts. However, the cleaved forms of NOTCH2 and NOTCH4 were consistently expressed among all cell lines. It is impossible to predict the status of cleaved NOTCH3 expression in these cells due to the lack of a specific antibody to test it. This data suggests that NOTCH1 signaling is over-activated in the resistant cells.

Upon NOTCH-ligand binding, NOTCH proteins exhibit a conformational change leading to S2 cleavage in the NOTCH extracellular domain by disintegrin and metalloprotease (ADAM) proteins (10). NOTCH S2 cleavage leads to a S3 cleavage by the gamma-secretase complex releasing the NOTCH intracellular domain (NICD) into the cytoplasm (11). To explore the implication of the ADAM family of proteins in the activation of Notch signaling, especially NOTCH1, in enzalutamide-resistant cells we tested the protein expression of ADAM9, ADAM10 and ADAM17 (TACE). ADAM9 is similarly expressed in LNCaP vs. MR49F and downregulated in C4-2R compared to C4-2 (Fig. 3D). TACE is up-regulated in both resistant cell lines compared to their sensitive counterparts. The active form of ADAM10 (lower band) is highly upregulated in MR49F and C4-2R compared to LNCaP and C4-2 respectively. These data indicate that upstream proteases, responsible for the activation of the Notch signaling pathway are predominantly upregulated in resistant cells, offering a potential explanation to the increased expression of cleaved-NOTCH1.

To explore a potential mechanism involved in the exclusive activation of NOTCH1, the highly activated ADAM10 was inhibited using GI254023X in MR49F and 22R1 (another enzalutamide-resistant cell line) cells. Upon treatment with GI254023X, cleaved-NOTCH1 expression is significantly reduced in 22R1 and MR49F (data not shown). However, the expression of cleaved NOTCH2 appears to be unaffected by
GI254023X treatment. This offers a potential explanation to the exclusive overexpression of cleaved-NOTCH1 compared to the expression of cleaved-NOTCH2 and -NOTCH4 in resistant cells.

After its internalization, the NICD translocates to the nucleus and activates the expression of Notch signaling downstream targets such as hairy and enhancer of split (HES) genes, c-MYC, and others (12). To confirm that Notch signaling is further activated in enzalutamide-resistant cells compared to enzalutamide-sensitive cells we tested the protein expression of HES1 and c-MYC. Both proteins are upregulated in MR49F and C4-2R compared to LNCaP and C4-2 respectively (Fig. 3D). This indicates that Notch signaling is activated in enzalutamide-resistant cells.

**Knockdown of NOTCH1 in C4-2R cells increases sensitivity to enzalutamide.** To test whether Notch1 signaling is directly involved in resistance, we designed and generated shRNA vectors targeting NOTCH1 and NOTCH2 in C4-2R cells. Figure 4A shows successful knockdown of NOTCH1 in C4-2R cells compared to cells infected with shCtrl virus. Exposure of C4-2R-shNOTCH1 cells to enzalutamide induces an increase in apoptosis represented by cleaved-PARP expression compared to C4-2R-shCtrl cells (Fig. 4B). Furthermore, exposure of C4-2R-shNOTCH1 cells to increasing concentrations of enzalutamide induces a decrease in cell proliferation (Fig. 4C). In contrast, enzalutamide didn’t induce the same effects on C4-2R-shNOTCH2 cells when compared to shCtrl (Figs. S1A, S1B and S1C). This suggests that knockdown of NOTCH1 is sufficient to reduce cell proliferation and increase apoptosis indicating a re-sensitization of C4-2R to enzalutamide.

**Inhibition of Notch signaling pathway re-sensitizes enzalutamide-resistant cells to enzalutamide and increases apoptosis and decreases colony formation ability.** To test whether inhibiting Notch signaling in prostate cancer enzalutamide-resistant cells re-sensitizes these cells to enzalutamide we treated MR49F, C4-2R and 22RV1 cells with enzalutamide, PF03084014 (gamma-secretase inhibitor) or both. Treatment with PF-03084014 induced a decrease in HES1 and c-MYC expression, indicating an inhibition of Notch signaling in all cell lines (Figs. S2A, S2B and S2C). Exposure to both PF-03084014 and enzalutamide induced an increase in cleaved-PARP expression levels indicating an increased apoptosis after 72 hours (Figs. 5A, 5C and 5E). In addition, in the same groups we observed a decrease in AR and PSA levels in MR49F and C4-2R compared to enzalutamide exposure alone (Figs. 5A and 5C). In 22RV1, the combination of enzalutamide and PF-03084014 caused a reduction in AR and AR-V7 protein levels (Fig. 5E). Furthermore, Figures 5B, 5D and 5F show a decrease in colony numbers and colony size in cells treated with both PF-03084014 and enzalutamide compared to cells treated with DMSO, enzalutamide or PF-03084014 alone. These results suggest that inhibition of Notch signaling re-sensitizes resistant cells to enzalutamide by blocking the AR signaling and reducing AR expression.

**PF-03084014 and enzalutamide treatment induces a decrease in 22RV1 tumor xenografts.** To further validate our in vitro data, we generated an enzalutamide-resistant mouse xenograft model using 22RV1 cells. Nude mice were castrated and inoculated subcutaneously with 22RV1 cells. Upon tumor formation mice were treated for 4 weeks with a vehicle, enzalutamide, PF-03084014 or the combination of enzalutamide and PF-03084014. Mice in the combination group showed a significant decrease in tumor weight compared to vehicle or single treatment groups (Figs. 6A and 6C). There were no significant signs of toxicity in mice. Mice from the combination group showed a significant decrease in body weight only when compared to mice treated with PF-03084014 alone (Fig. 6B).

Immunofluorescent staining of tumors showed effective inhibition of the Notch signaling pathway by PF-03084014 represented by a significant decrease in the percentage of HES1 expressing cells in the PF-03084014 and combination treatment groups (Fig. S2D). In addition, a significant increase in cleaved-Caspase 3 expressing cells in the combination treatment group compared to the other groups indicated an increase in apoptosis.
(Figs. 6D and 6E). KI-67 staining was significantly decreased in tumors from the combination treatment group compared to the other groups, suggesting a reduced proliferation (Figs. 6D and 6F). Combination of enzalutamide and PF-03084014 induced a significant decrease in AR expressing cells within the tumor (Figs. 6D and 6G). These results suggest that blocking Notch signaling in vivo re-sensitizes 22RV1 cells to enzalutamide by increasing apoptosis, reducing cell proliferation and AR expression.

**Discussion**

The already FDA-approved enzalutamide is currently under investigation in clinical trials with early stage prostate cancer patients. If the outcome of these trials turns out to be a success, enzalutamide will become widely used for treatment of prostate cancer patients at all stages of the disease. However, one of the major obstacles is development of enzalutamide resistance in patients. Most of therapy-resistance studies in prostate cancer focus on the role of the AR signaling pathway in overcoming the resistance to ADT and enzalutamide (13). In this study, our aim was to identify pathways that are independent of the AR signaling pathway and may play a role enzalutamide resistance. We compared enzalutamide-resistant cells MR49F and C4-2R to their enzalutamide-sensitive counterparts LNCaP and C4-2, respectively. Our analysis revealed hundreds of genes that are up- or down-regulated in the enzalutamide-resistant cells (Fig. 1). The combination of our RNA-seq with patient sample bioinformatics analysis helped identify Notch signaling pathway as a potential deregulated pathway in enzalutamide-resistant cells.

In prostate cancer, studies have shown contradicting roles of NOTCH signaling where it either plays a tumor suppressive or a tumor promoting role (14, 15). Our results indicate that the Notch signaling pathway plays a tumor promoting role in prostate cancer by contributing to enzalutamide-resistance and promoting cell survival. Furthermore, NOTCH proteins have been shown to contribute to therapy resistance in prostate cancer (16). In addition, studies have shown synergistic positive results in prostate cancer cells when Notch signaling inhibitors were combined with androgen deprivation therapies (ADTs) (17, 18). Our data supports the observations above where we show that Notch signaling is activated in enzalutamide-resistant cells and inhibition of this pathway restores sensitivity to enzalutamide.

In addition, Notch signaling has been shown to be a driver of stemness and contributor to survival and maintenance of cancer stem cells, which are believed to a driver of cancer therapy resistance (15, 19). There is evidence that ADT and enzalutamide treatment promote the acquisition of stem cell-like features in prostate cancer cells (20, 21). Deregulation of Notch signaling in enzalutamide-resistant cells may play a role in maintenance of stem-like features leading to therapy resistance.

To investigate whether Notch signaling is active in resistant cells, expression of cleaved-Notch isoforms was tested. Our data suggests that Notch1 signaling is active due to a high expression of cleaved-NOTCH1 in the resistant cells (Fig. 3B). Furthermore, downstream targets such as HES1 and c-MYC were overexpressed in resistant cells indicating an active pathway (Fig. 3C). However, due to the lack of a specific cleaved-NOTCH3 antibody we were unable to verify the expression of this protein. Thus, Notch3 may also be playing a role in the activation of downstream targets.

To further understand how Notch signaling is activated in enzalutamide-resistant cells we investigated the expression of upstream proteases involved in Notch receptor activation and downstream targets of the pathway. Our results show that ADAM10 is activated and TACE is upregulated in the resistant cells (Fig. 3C). ADAM10 and TACE overexpression contributes to therapy resistance and their inhibition leads to re-sensitizing tumor cells to existing therapies in different cancers (22–24). Here, we believe that the overexpression of the metalloproteases might be involved in the activation of the Notch1 signaling pathway. According to our data, inhibition of
ADAM10 with GI254023X reduced the expression of cleaved-NOTCH1 exclusively (Fig. 3D), which suggests a higher sensitivity of Notch1 cleavage to changes in ADAM10 expression or activation. Other explanations may be attributed to selectivity in ligands towards Notch1 over other isoforms (25) and the elevated number of reported mutations in the heterodimerization domain of Notch1 that can affect its ligand independent cleavage compared to the other isoforms (26).

Notch1 signaling is shown to become activated and to play a role in cell death and drug-sensitivity after cells acquire resistance in other cancers (27, 28). To investigate whether Notch1 signaling plays a significant role in enzalutamide resistance we generated shNotch1 C4-2R cells. These cells have a better response to enzalutamide treatment compared to shCtrl cells (Fig.4). In contrast, knockdown of NOTCH2 in C4-2R didn’t yield the same results.

Inhibitors and blockers of Notch signaling are being investigated in clinical trials in multiple cancers. To investigate whether blockade of Notch signaling pathway reverses enzalutamide-resistance in MR49F, C4-2R and 22RV1, we used the gamma secretase inhibitor PF-03084014. Combination of enzalutamide and PF-03084014 showed overexpression of cleaved PARP expression in all cell lines (Figs. 5A, 5C and 5E). One of the established mechanisms of resistance to ADT is increased AR and AR-variants expression in prostate cancer cells (5, 6). Our data indicate that exposure to enzalutamide and PF-03084014 decreased full length AR, PSA and AR-V7 expression, potentially explaining the restoration of enzalutamide function in the resistant cells (Figs. 5A, 5C and 5E). Additionally, increased levels of c-MYC has been shown to promote ligand-independent prostate cancer survival (29). Our data show that combination of enzalutamide and the gamma secretase inhibitor decreases c-MYC expression in the resistant cells offering another mechanism by which resistance is reversed in those cells (Figs. S2A, S2B and S2C). Furthermore, the inhibition of AR and Notch signaling together induced a significant decrease in colony formation ability of MR49F, C4-2R and 22RV1 (Fig.5 B, D and F). In the xenograft model, the combination therapy showed growth inhibition in tumors compared to vehicle and single treatment groups, with no signs of significant toxicity. This observation was accompanied by an increase in cleaved-Caspase-3 expression, a decrease in KI-67 expression and a decrease in AR expressing cells which replicated our in vitro cell line data (Fig. 6).

Finally, our study adds up to existing data on the oncogenic role of Notch signaling in prostate cancer. Notch1 signaling is activated in enzalutamide-resistant cells, and inhibition of this pathway may restore enzalutamide function in vitro and in vivo, leading to a better outcome. These data offer a precedent for the combination of Notch signaling inhibitors with enzalutamide and potentially other ADTs.

**Materials and Methods**

**Mammalian cell lines:**

LNCaP, C4-2 and 22RV1 cells were purchased from ATCC in 2016. MR49F and C4-2R cells were kindly provided by Dr. Amina Zoubeidi (5) and Dr. Allen Gao (6). LNCaP and C4-2 were used as enzalutamide-sensitive cell lines. Whereas, MR49F, C4-2R and 22RV1 were considered enzalutamide-resistant cell lines. For this study LNCaP and C4-2 were paired with their enzalutamide-resistant counterparts MR49F and C4-2R, respectively. 22RV1 was used as an independent cell line with no enzalutamide-sensitive pair. All cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) Fetal Bovine Serum and 1% (v/v) Penicillin/Streptomycin at 37°C in a humidified incubator with 5% carbon dioxide. MR49F and C4-2R were constantly maintained in medium supplemented with 10 and 20 µM enzalutamide, respectively.
Inhibitors:
PF-03084014 and enzalutamide were purchased from Selleckchem. GI254023X was purchased from Sigma-Aldrich. For in vitro experiments, PF-03084014, enzalutamide and GI254023X were suspended in DMSO. For in vivo experiments, PF-03084014 was suspended in 0.5% Methylcellulose and enzalutamide was formulated in 5% DMSO and 0.1% Tween80 suspended in 1% carboxymethyl cellulose.

Western blot:
Cells were harvested and washed with PBS. Pellets were resuspended in TBSN buffer (20 mmol/L Tris-HCl, pH 8.0, 0.5% NP-40, 5 mM EGTA, 1.5 mmol/L EDTA, 0.5 mM sodium vanadate and 150 mM NaCl) supplemented with protease and phosphatase inhibitor cocktail and subjected to sonication. Sample protein concentrations were measured using Pierce BCA Protein Assay Kit. Equal amounts of proteins were mixed with 4x SDS loading buffer and loaded onto SDS-PAGE gels. Upon protein separation, proteins were transferred to PVDF membranes and subjected to detection using appropriate primary and secondary antibodies. GAPDH and β-Actin were used as housekeeping genes.

RNA-sequencing:
RNA was extracted from LNCaP, MR49F, C4-2, C4-2R after 4-hour treatment with enzalutamide using RNeasy kit (QIAGEN). Libraries were prepared using TruSeq Stranded kit (Illumina) at the Purdue Genomics Facility. 2x100 bp reads were sequenced in 2 lanes using the HiSeq2500 on high-output mode. Before library preparation the dscDNA quality was checked using an Agilent Bioanalyzer with the High Sensitivity DNA Chip. All plots were checked to ensure that read quality for the reads that would be used in the remainder of the analysis were of high quality and with no problems. Tophat2 (30, 31) was used to align reads to the Ensembl Homo sapiens genome database version GRCh38.p5. The mitochondrial chromosome and the nonchromosomal sequences were excluded from the analysis. The htseq-count script in HTSeq v.0.6.1 (32) was run to count the number of reads mapping to each gene. DESeq2 (33), edgeR (34) and Cufflinks2 (35, 36) were used for differential expression analysis.

Gene Set Enrichment Analysis:
Clinical data was collected from Level 3 (for Segmented or Interpreted Data, IlluminaHiSeq_RNASeqV2) of the TCGA database. Samples were divided into low-grade (Gleason score <8) and high-grade (Gleason score ≥8). Of the high-grade samples, we looked at 72 out of 497 cases that had been treated with anti-hormone therapy. We then separate the 72 patients into AR high and AR low based on the expression of the genes from the AR signaling pathway. AR high cases were considered to mimic enzalutamide-resistant cells and AR low cases were considered to mimic enzalutamide-sensitive cells. Using GSEA we detected pathway enrichment in AR high (26 samples) vs AR low (46 samples).

Colony formation assay:
Cells were seeded at equal densities in 6-well plates, followed by treatment with DMSO as a control, PF-03084014, enzalutamide or the combination of both. After colonies became of size, cells were subjected to fixation with 4% Paraformaldehyde followed by staining with 0.5% crystal violet solution.

MTT Cell Proliferation Assay:
To evaluate cell proliferation, cells were seeded in 96-well plates and treated with increasing concentrations of enzalutamide. At the end of incubation time, cells were then treated with 0.5 mg/ml MTT (3-(4, 5-dimethylthiazoyl-2)-2,5-diphenyltetrazolium bromide) for an hour. Supernatant was removed and DMSO was added to dissolve crystals. Optical density was measured at a wavelength of 560 nm. Values were normalized as 100% to the control group treated with DMSO.
**Lentivirus production and Notch1 knockdown in C4-2R cells:**

A lentivirus system encoding shRNA targeting a scrambled sequence, *NOTCH1* and *NOTCH2* mRNA was used for knockdown. Sequences targeting Ctrl 5’-TTCTCCGAAACGTGTACGTCG-3’, *NOTCH1* 5’-GCATGTGTAACATCAACAT-3’, and *NOTCH2* 5’-CAGGTAGCTCAGACCATT-3’ were cloned in pLVshRNA-puro. Lentiviral particles were generated by transfecting shCtrl, sh*NOTCH1*, sh*NOTCH2* with psPAX2 and PMD.2G plasmids at a ratio of 4:3:1 in 293T cells. Medium was collected and used to infect C4-2R cells with corresponding shRNA to generated C4-2R-shCtrl, C4-2R-sh*NOTCH1* and C4-2R-sh*NOTCH2*.

**22RV1 mouse xenografts:**

Castrated nu/nu mice were subjected to a right flank subcutaneous inoculation of 2 x 10⁵ cells 22RV1 cells suspended in PBS and mixed with Matrigel (1:1). Tumor-bearing mice were randomly assigned into 4 groups (n=4) where mice from different groups received through oral gavage a vehicle control, 20 mg/kg/day of enzalutamide, 110 mg/kg of PF-03084014 daily on alternate weeks, or the combination of both. Upon necropsy tumors were collected and weighed. *In vivo* experiments were approved by the Purdue Animal Care and Use Committee.

**Immunofluorescent chemistry:**

Xenograft tumors were fixed in 4% neutral buffered paraformaldehyde, sectioned and stained using specific antibodies. Staining was accomplished with the M.O.M.TM kit from VECTOR LABORATORIES. Positively stained cells were quantified using NIS-Elements AR 3.2. All groups were normalized to vehicle control.

**Statistical analysis:**

Standard 2-tailed Student t tests were performed to analyze statistical significance of the results. Two-way ANOVA tests were performed to analyze statistical significance of datasets with grouped observations. One-way ANOVA test was performed to analyze statistical significance of immunofluorescence quantification. A P-value of less than 0.05 indicates statistical significance.

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**Conflict of interest**

The authors declare no conflicts of interest.
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Figure 1. Long-term enzalutamide treatment of prostate cancer cell lines induces global gene expression changes after acquiring resistance. A, Heatmap showing the Euclidian distances between samples, made with the DESeq2 transformed data after a regularized log transformation was performed. B, PCA plot of libraries (salmon colored points represent C4-2 samples, green represent C4-2R samples, teal represent LNCaP samples, and purple represent MR49F samples.). Overall 54% of the variance is captured by the first principle component (shown on the x-axis) and principle component two (shown on the y-axis) accounts for 28% of the variation. Data was normalized in DESeq2 and a regularized log transformation performed prior to doing the PCA. C, Smear plots from edgeR showing, in red, transcripts differentially expressed (FDR < 0.01) between, respectively, enzalutamide-resistant/enzalutamide-sensitive cells. Blue lines indicate 2-fold-change cutoff. The x-axis shows the average log(count per million) and the y-axis shows the log2(fold change). D, Venn diagrams of overlap between differential expression results amongst the three statistical packages Cufflinks, edgeR and DESeq2 for both comparisons.
Figure 2. Notch signaling pathway is enriched in patient samples that mimic enzalutamide-resistant cell lines. **A**, Boxplot of normalized AR counts for all samples using DESeq2. **B**, Heatmap representing a cohort of patients from the TCGA database, separated into samples with high AR expression (26 samples) and samples with low AR expression (46 samples). **C**, GSEA enrichment plot of the Reactome pre-Notch transcription and translation gene set in the cohort of patient with AR low vs. AR high. The plot indicates a negative enrichment, meaning that the queried gene set correlates with AR high samples. **D**, GSEA enrichment plot of the Reactome pre-Notch expression and processing gene set in the cohort of patient with AR low vs. AR high. The plot indicates a negative enrichment, meaning that the queried gene set correlates with AR high samples. **E**, NOTCH2 gene expression analysis in the cohort of patients and its correlation with AR gene expression indicates a strong positive correlation with a correlation coefficient of $r=0.4331$. **F**, c-MYC gene expression analysis in the cohort of patients and its correlation with AR gene expression indicates a strong positive correlation with a correlation coefficient of $r=0.4536$. **G and H**, NOTCH2 and c-MYC counts matched to the samples number from the cohort of patients.
Figure 3. Expression of Notch signaling pathway genes is deregulated in enzalutamide-resistant cells. A, Heatmap representing gene expression patterns of Notch signaling pathway genes in LNCaP vs MR49F cell lines. B, Heatmap representing gene expression patterns of Notch signaling pathway genes in C4-2 vs C4-2R cell lines. C and D, LNCaP, MR49F, C4-2 and C4-2R cells were treated with enzalutamide for 4 hours and subjected to western blot analysis. E, 22RV1 cells were treated with GI254023X (ADAM 10 inhibitor) for 48 hours and subjected to western blot analysis.

Figure 4. Knockdown of NOTCH1 induces an increase in cell death and a decrease in cell proliferation. A, C4-2R cells were infected with shCtrl or shNOTCH1 virus particles and selected with puromycin. Cells were collected and subjected to western blot analysis with indicated antibodies. B, Stable C4-2R shCtrl and shNOTCH1 cells were treated with DMSO or 40 µM enzalutamide for 72 hours and subjected to western blot analysis. C, Stable C4-2R shCtrl and shNOTCH1 cells were treated with DMSO or increasing
concentrations of enzalutamide and subjected to MTT assay. Optical densities were measured and normalized to DMSO-treated samples. * indicates that p-value<0.05 and ** indicates that p-value<0.01.
Figure 5. Inhibition of Notch signaling pathway re-sensitizes enzalutamide-resistant cells to enzalutamide and causes cell death and decreased colony formation ability. **A, C and E,** MR49F, C4-2R and 22RV1 cells were treated with DMSO, PF-03084014, enzalutamide or both at the indicated concentrations for 72 hours. Cells were collected and subjected to western blot analysis. **B, D, and F,** Low seeding density MR49F, C4-2R and 22RV1 cells were treated with DMSO, PF-03084014, enzalutamide or both at the indicated concentrations until colonies were formed. Cells were fixed with 4% Formaldehyde and stained with crystal violet.
**A**
Tumor weight (g)

- Vehicle
- Enzalutamide 25mg/kg/d
- Enzalutamide + PF-03084014

**B**
Body weight (g)

- Vehicle
- Enzalutamide 25mg/kg/d
- Enzalutamide + PF-03084014

**C**

- Vehicle
- Enzalutamide
- PF-03084014
- Enzalutamide + PF

**D**

|          | DAPI | c-Caspase-3 | Merge | DAPI | KI-67 | Merge | DAPI | AR | Merge |
|----------|------|-------------|-------|------|-------|-------|------|----|-------|
| Vehicle  |      |             |       |      |       |       |      |    |       |
| Enzalutamide |      |             |       |      |       |       |      |    |       |
| PF-03084014 |      |             |       |      |       |       |      |    |       |
| Enzalutamide + PF-03084014 |      |             |       |      |       |       |      |    |       |

**E**

- Normalized % of c-Caspase-3 positive cells

|          | Vehicle | Enzalutamide 25mg/kg/d | PF-03084014 110mg/kg | Enzalutamide + PF-03084014 |
|----------|---------|------------------------|-----------------------|-----------------------------|

**F**

- Normalized % of KI-67 positive cells

|          | Vehicle | Enzalutamide 25mg/kg/d | PF-03084014 110mg/kg | Enzalutamide + PF-03084014 |
|----------|---------|------------------------|-----------------------|-----------------------------|

**G**

- Normalized % of AR positive cells

|          | Vehicle | Enzalutamide 25mg/kg/d | PF-03084014 110mg/kg | Enzalutamide + PF-03084014 |
|----------|---------|------------------------|-----------------------|-----------------------------|
Figure 6. PF-03084014 combined with enzalutamide treatment induces a decrease in enzalutamide-resistant tumor xenograft. A and B, 22RV1 xenograft mice were treated with a vehicle control, enzalutamide, PF-03084014 or a combination of enzalutamide and PF-03084014. Upon completion, tumor weight and body weight were measured. (n=4 mice) C, Representative image of tumors from different treatment groups. D, Immunofluorescent images from tumor sections stained with cleaved-Caspase3, KI-67, AR and DAPI. E, F and G, Quantification of cleaved-Caspase3, KI-67 and AR positively stained cells (n=3). All groups were normalized to the vehicle control group. * indicates that p-value<0.05, ** indicates that p-value<0.01 and *** indicates that p-value<0.001.

Table 1

| MR49F vs LNCaP, 1% FDR, 2FC cutoff | Total | Up in Resistant Cells | Down in Resistant Cells |
|------------------------------------|-------|-----------------------|------------------------|
| DESeq2                             | 1,627 | 738                   | 889                    |
| edgeR                              | 2,520 | 1,026                 | 1,294                  |
| Cufflinks                          | 1,279 | 719                   | 560                    |

Table 2

| C4-2R vs C4-2, 1% FDR, 2FC cutoff | Total | Up in Resistant Cells | Down in Resistant Cells |
|-----------------------------------|-------|-----------------------|------------------------|
| DESeq2                            | 2,331 | 1,206                 | 1,125                  |
| edgeR                             | 3,121 | 1,584                 | 1,567                  |
| Cufflinks                         | 1,737 | 871                   | 866                    |

Table 1 and 2. Total, up-regulated and down-regulated transcripts differentially expressed (FDR < 0.01 and 2-fold cutoff) between, respectively, enzalutamide-resistant/enzalutamide-sensitive cells using the 3 different packages Cufflinks, edgeR, and DESeq2.
NOTCH signaling is activated in and contributes to resistance in enzalutamide-resistant prostate cancer cells

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