Figures and figure supplements

Aberrant corticosteroid metabolism in tumor cells enables GR takeover in enzalutamide resistant prostate cancer

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Figure 1. GR stimulation with enzalutamide resistance in prostate cancer is tightly regulated by glucocorticoid metabolism in target tissues. (A) Glucocorticoid metabolism in target tissues. Stimulation of GR by cortisol in humans is limited by 11β-HSD2, which oxidizes and converts cortisol to cortisone. Figure 1 continued on next page
inactive cortisone. In mice, 11β-HSD2 converts active corticosterone to inactive 11-dehydrocorticosterone. (B) Enzalutamide (Enz) sustains cortisol levels by retarding inactivation in the LAPC4 and VCaP human prostate cancer cell lines. Cells were treated with the indicated concentrations of Enz or vehicle for 36 days (LAPC4) or 40 days (VCaP), and subsequently treated with [3H]-cortisol (100 nM) for the indicated times, followed by steroid extraction from media (above) and cells (below), steroid separation and quantitation with HPLC. The experiment was done in duplicate and repeated at least three times. (C) Cortisol inactivation is impaired in xenograft tumors treated with Enz. Fresh tumor tissues were harvested from LAPC4 or VCaP xenografts grown in orchiectomized mice and treated with Enz or chow alone (n = 5 tumors per treatment group). Tumors were treated with [3H]-cortisol (100 nM) for the indicated times and steroids were extracted from media and analyzed by HPLC. Error bars represent the SD. (D) Enz suppresses LAPC4 cell line proliferation. LAPC4 cells were treated with vehicle (Ctrl) or the indicated concentration of Enz for the designated number of days and cell viability was assessed using CellTiter-Glo. Cell viability was normalized to day 0, experiments were performed in triplicate and error bars represent the SD.

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**Figure 1—figure supplement 1.** Effects of Enz on LAPC4 cells. (A) Short term Enz treatment does not affect cortisol metabolism. Previously untreated cells were treated with the indicated concentration of Enz or Vehicle and concomitantly with [3H]-cortisol (100 nM) for the indicated times and steroids.

**Figure 1—figure supplement 1 continued on next page**
were separated and quantitated by HPLC. (B) Enz suppresses expression of AR-regulated transcripts and has no acute effect on expression of GR, HSD11B2 or HSD11B1. LAPC4 cells were treated with the indicated concentration of Enz or Vehicle for 24 hr and the indicated transcripts were assessed by qPCR. Expression is normalized to Vehicle control and RPLP0. (C) The viability of LAPC4 cells recovers with long-term Enz treatment. The cells were treated with long-term (Enz D56), short-term (Ctrl + Enz) Enz (10 μM), or no treatment (Ctrl) for the indicated number of days and cell viability was assessed relative to day 0. Experiments were performed in triplicate and error bars represent the SD.

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Figure 2. Enzalutamide promotes 11β-HSD2 protein loss in cell line models and tissues from patients with prostate cancer. (A) Enzalutamide (Enz) treatment results in the loss of 11β-HSD2 protein that occurs concurrently with an increase in GR protein in the LAPC4 model of CRPC as assessed by Western blot. (B) 11β-HSD2 protein expression in Enz treated LAPC4 cells as assessed by immunocytochemistry. (C,D) Loss of 11β-HSD2 and increase in GR protein in VCaP model of CRPC as assessed by Western blot. (E) Western blot analysis of 11β-HSD2 and GR expression in patient samples. Figure 2 continued on next page.
in GR protein similarly occur with Enz treatment in the VCaP and MDA-PCa-2b models. (E) Enz induces loss of 11β-HSD2 protein in tissue from patients with prostate cancer. Local prostate biopsies were obtained from Patients #1 and #2 with image guidance in a neoadjuvant study before (Pre) and after (Post) two months of treatment with Enz and medical castration. Patients #3 and #4 had biopsies of metastatic CRPC from lymph nodes before and after three months (Patient #3) and 11 months (Patient #4) of treatment with Enz. Fresh tissues from Patients #5-#11 were obtained from surgical prostatectomy specimens and incubated with vehicle or Enz (10 μM) for 7–8 days prior to protein extraction and Western blot.

DOI: 10.7554/eLife.20183.004
Figure 2—figure supplement 1. Response to Enz in prostate cancer cell lines and human tissues. (A) Enz treatment does not change 11β-HSD2 protein expression in the AR-negative DU145 prostate cancer cell line. (B) Enz treatment does not change 11β-HSD1 protein expression in AR-negative LAPC4 prostate cancer cell line. (C) Enz treatment does not change 11β-HSD1 protein expression in AR-negative VCaP prostate cancer cell line. (D) Enz treatment does not change 11β-HSD2 protein expression in AR-negative MDA-Pca-2b prostate cancer cell line. (E) Enz treatment does not change 11β-HSD2 protein expression in AR-negative LAPC4 prostate cancer cell line. (F) Patient samples
Figure 2—figure supplement 1 continued

expressing prostate cancer cell lines. Cells were treated with the indicated concentrations of Enz, whole cell protein lysates were obtained, separated and assessed with anti-11β-HSD1 and anti-β-actin antibodies. (C) GR transcript increases and HSD11B2 is unchanged with long-term Enz treatment of LAPC4 and VCaP cells. Expression is normalized to vehicle treated cells and RPLP0. (D) Enz does not directly antagonize 11β-HSD2. LAPC4 cells were transfected with a vector encoding 11β-HSD2, in the presence of the indicated concentration of Enz or Vehicle, and conversion from [3H]-cortisol (100 nM) to cortisone was assessed by HPLC. Experiments performed in biological duplicate. (E) 11β-HSD2 loss is not attributable to GR stimulation. LAPC4 cells were treated with dexamethasone (DEX; 100 nM) for the indicated durations, whole cell protein lysates were obtained and assessed with anti-11β-HSD2 and anti-β-actin antibodies. (F) GR protein expression is induced in a subset of the patient tissues from Figure 2E. All six tissues that have induction of GR expression exhibit loss of 11β-HSD2 in Figure 2.

DOI: 10.7554/eLife.20183.005
Figure 3. 11β-HSD2 expression reverses enzalutamide-sustained cortisol levels and GR-responsive gene expression. (A) Impeded conversion from cortisol to cortisone with Enz treatment is reversible with transient and (B) stable 11β-HSD2 expression. Cells expressing 11β-HSD2 or empty vector...
Figure 3 continued

(control) were treated with the indicated concentration of Enz for 40 days, followed by treatment with [3H]-cortisol and analysis of steroids in media by HPLC. (C) With Enz treatment, only cortisol-induced GR signaling is specifically reversible with forced stable 11β-HSD2 expression. LAPC4 cells were treated with Enz for 36 days, starved with phenol-red-free medium containing 5% Charcoal:Dextran-stripped FBS for 48 hr and transfected with a plasmid expressing 11β-HSD2 and treated with the indicated conditions for 24 hr. Only cortisol induction of PSA expression, which is GR- and metabolism-dependent, is reversible by 11β-HSD2. Expression of KLF9, which is regulated only by GR, is induced by cortisol and dexamethasone, but only cortisol induction is reversible by 11β-HSD2. Expression of PMEPA1, which is regulated only by AR, is induced with DHT only and not reversible by 11β-HSD2. Expression is normalized to vehicle-treated cells (not shown) and RPLP0 expression. The experiment was performed four times. Error bars represent the SD of a representative experiment performed in triplicate.

DOI: 10.7554/eLife.20183.006
11β-HSD2 overexpression (OE) in the long-term Enz-treated LAPC4 cells is comparable to endogenous expression in the human placental derived JEG-3 cell line. 

DOI: 10.7554/eLife.20183.007
**Figure 4.** Reinstatement of 11β-HSD2 expression restores sensitivity to enzalutamide therapy by specifically suppressing tumor corticosterone. (A) Expression of 11β-HSD2 reverses enzalutamide (Enz) resistant LAPC4 CRPC xenograft tumor growth. (B) Progression-free survival is prolonged by 11β-HSD2 expression.

**Vector/Enz 11β-HSD2/Enz**

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**Tumor**

**Serum**

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HSD2 expression in Enz-treated LAPC4 xenografts. N.S. = not significant. (C) 11β-HSD2 expression reverses Enz resistance in the VCaP xenograft model of CRPC as assessed by decreased tumor volume and (D) prolongation of progression-free survival. For both xenograft studies, cells expressing 11β-HSD2 or vector (control) were grown in orchiectomized mice supplemented with DHEA and arbitrarily assigned to Enz or chow (Ctrl). For the comparisons in tumor volume, the significance of the difference between 11β-HSD2/Enz and Vector/Enz was calculated with an unpaired and two-tailed t-test on day 24 (LAPC4) or day 23 (VCaP). For the comparisons in progression-free survival, the significance of the difference between 11β-HSD2/Enz and Vector/Enz was calculated with a log-rank test. (E) The absolute concentration of corticosterone is reduced in xenograft tumors expressing 11β-HSD2. (F) The percentage of corticosterone relative to 11-dehydrocorticosterone is reduced in tumors expressing 11β-HSD2. (G) The absolute concentration of corticosterone and (H) percentage of corticosterone relative to 11-dehydrocorticosterone in serum are unaffected in mice harboring tumors with restored 11β-HSD2 expression. P values in E-H were calculated with an unpaired and two-tailed t-test.

DOI: 10.7554/eLife.20183.008
Forced 11β-HSD2 expression in Enz-treated LAPC4 xenografts is comparable to endogenous expression in the MDA-PCA-2b prostate cancer cell line and the human placental derived JEG-3 cell line.

DOI: 10.7554/eLife.20183.009
Figure 5. AMFR is required for 11β-HSD2 ubiquitination and the enzalutamide-induced metabolic phenotype that sustains local cortisol concentrations and enzalutamide-resistance. (A) 11β-HSD2 and AMFR co-immunoprecipitate. Immunoprecipitation (IP) and immunoblot (IB) from endogenously
expressed proteins in whole cell protein lysate from LAPC4 cells were performed with the indicated antibodies. The experiment was performed twice. 
(B) AMFR promotes 11\(\beta\)-HSD2 ubiquitination. Proteins were expressed in 293 cells, proteins tagged with ubiquitin-His, were pulled-down with Ni-agarose beads, and immunoblot was performed with the indicated antibodies. The experiment was performed twice. (C) Silencing AMFR expression with two independent shRNAs increases 11\(\beta\)-HSD2 protein. LAPC4 cells stably expressed shRNAs against AMFR (shAMFR) or non-silencing control expression vector. The experiment was performed three times. (D) Blockade of Enzalutamide (Enz)-mediated 11\(\beta\)-HSD2 loss by silencing AMFR reverses the metabolic phenotype that confers sustained cortisol concentrations. Cells were treated with the indicated concentration of Enz and subsequently were treated with \(^{3}\text{H}\)-cortisol (100 nM) for the indicated times, followed by steroid extraction from media and cells, and steroid analysis by HPLC. Error bars represent the SD of biological triplicates. The experiment was performed three times. Enz treatment in panel D was for 38–42 days. (E) AMFR is required for tumor growth through enzalutamide therapy. Xenografts from LAPC4 cells expressing shAMFR or non-silencing control vector (shCtrl) were grown in surgically orchiectomized mice supplemented with DHEA and treated with Enz when tumors reached 100 mm\(^3\). The significance of the difference between shCtrl and shAMFR groups was calculated with an unpaired and two-tailed t-test on day 20. (F) Progression-free survival is increased in tumors lacking AMFR. The significance of the difference between shCtrl and shAMFR groups was determined with a log-rank test. (G) Xenograft tumors with genetic ablation of AMFR retain 11\(\beta\)-HSD2 protein expression. Xenograft tissues were collected at the end of the xenograft study and immunoblot was performed with the indicated antibodies.

DOI: 10.7554/eLife.20183.010
Figure 5—figure supplement 1. AMFR and Erlin-2 regulation and cortisol metabolism with Enz treatment. (A) Erlin-2 but not AMFR is consistently upregulated with Enz treatment of LAPC4 cells. (B) Erlin-2 is up-regulated in 8 of 11 human prostate tissues. Immunoblots were performed as described...
previously. (C) Erlin-2 overexpression (OE) suppresses expression of 11β-HSD2 protein in LAPC4 cells and Erlin-2 knockdown by siRNA increases 11β-HSD2 expression and activity in the long-term Enz-treated LAPC4 cells. (D) AMFR silencing does not regulate HSD11B2 transcript. qPCR was performed in triplicate and expression is normalized to shControl-expressing cells and RPLP0. (E) Reversal of the metabolic phenotype that sustains cortisol with Enz treatment by AMFR knockdown is reversed again by 11β-HSD2 knockdown (compare cortisol at 24 hr in shAMFR groups between siCTRL and siHSD11B2). The specificity of siHSD1B2 is shown by qPCR and immunoblot. LAPC4 cells stably expressing stably shCTRL or an shAMFR construct were treated with Enz as described for Figure 5, transiently transfected with siHSD11B2 or siCTRL and treated with [3H]-cortisol (100 nM). Experiments were performed in duplicate.

DOI: 10.7554/eLife.20183.011