Loss of dihydrotestosterone-inactivation activity promotes prostate cancer castration resistance detectable by functional imaging

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Androgens such as testosterone and dihydrotestosterone are a critical driver of prostate cancer progression. Cancer resistance to androgen deprivation therapies ensues when tumors engage metabolic processes that produce sustained androgen levels in the tissue. However, the molecular mechanisms involved in this resistance process are unclear, and functional imaging modalities that predict impending resistance are lacking. Here, using the human LNCaP and C4-2 cell line models of prostate cancer, we show that castration treatment—sensitive prostate cancer cells that normally have an intact glucuronidation pathway that rapidly conjugates and inactivates dihydrotestosterone and thereby limits androgen signaling, become glucuronidation deficient and resistant to androgen deprivation. Mechanistically, using CRISPR/Cas9-mediated gene ablation, we found that loss of UDP glucuronosyltransferase family 2 member B15 (UGT2B15) and UGT2B17 is sufficient to restore free dihydrotestosterone, sustained androgen signaling, and development of castration resistance. Furthermore, loss of glucuronidation enzymatic activity was also detectable with a nonsteroid glucuronidation substrate. Of note, glucuronidation-incompetent cells and the resultant loss of intracellular conjugated dihydrotestosterone were detectable in vivo by 18F-dihydrotestosterone PET. Together, these findings couple a mechanism with a functional imaging modality to identify impending castration resistance in prostate cancers.

The long-standing standard treatment for advanced prostate cancer is androgen deprivation therapy by way of medical or surgical castration (1). Therapeutic responses usually occur and tumors eventually develop resistance as castration-resistant prostate cancer (CRPC)2 (2, 3). This resistant state is frequently first heralded by a rising prostate-specific antigen (PSA), which is an androgen-responsive gene and is indicative of a reactivation of androgen receptor (AR) stimulation (4). CRPC is also accompanied by tumors developing the metabolic capability of regenerating their own potent androgens, i.e. testosterone and/or dihydrotestosterone (DHT), from extragonadal precursor steroids (5–7). The role and requirement for extragonadal androgen synthesis in advanced prostate cancer was recently bolstered by a profound prolongation of survival in clinical trials of patients treated upfront with extragonadal androgen synthesis inhibition in addition to castration (8, 9).

The metabolic mechanisms that enable sustained tissue concentrations of potent androgens in CRPC that occurs alongside the absence of gonadal testosterone in circulation remain poorly understood. Furthermore, imaging for prostate cancer is very limited and mainly focused on tumor localization. There is a complete absence of functional imaging approaches that are informative of the metabolic state of the disease that provides information on tumor susceptibility to systemic therapies in the clinical standard of care.

Glucuronidation is a major mechanism of androgen inactivation that normally limits AR stimulation and downstream signaling in peripheral tissues, including prostatic tissues (10). The 17β-OH group of testosterone and DHT make them substrates for UDP-glucuronosyltransferase (UGT) enzymes to form their respective inactivated glucuronide metabolites. In humans, testosterone is glucuronidated primarily by UGT2B7 and DHT, and DHT is glucuronidated by UGT2B7 and is marked by loss of glucuronidation enzymatic activity occurs in CRPC, protects intratumoral testosterone and/or DHT from its normal mechanism of tissue inactivation, and allows androgens from extragonadal origins to be sustained, thus enabling persistent AR signaling. We also show that positron-emission tomography (PET) imaging can be used to distinguish glucuronida-

2 The abbreviations used are: CRPC, castration-resistant prostate cancer; PSA, prostate-specific antigen; AR, androgen receptor; DHT, dihydrotestosterone; UGT, UDP-glucuronosyltransferase; DHEA, dehydroepiandrosterone; CSA, cyclosporin A; CSS, charcoal-stripped serum; qPCR, quantitative PCR; CT, computed tomography; Chemotherapy, CT, control.
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Results
Loss of glucuronidation activity occurs with a classical model of “androgen independence”

The LNCaP human cell line model of prostate cancer and its C4-2 derivative, which was developed as a resistant model by repeatedly propagating LNCaP through castrated mice (13), probably represent the most widely utilized human matched models of castration-sensitive (formerly known as “androgen-sensitive”) and castration-resistant (formerly known as “androgen-independent”) prostate cancer. Development of resistance by way of a truly androgen-independent mechanism would be expected to occur without the acquisition of an androgen-sustaining metabolic phenotype. In contrast, we found that although the LNCaP model normally rapidly inactivates DHT by glucuronidation, the C4-2 model has completely lost this enzymatic activity. For example, 3 h after DHT treatment, all DHT in media is glucuronidated in the LNCaP model and all detectable DHT remains free in the C4-2 model (Fig. 1A). This results in a marked prolongation of cellular exposure to DHT in media (Fig. 1B) and as well as in the intracellular space (Fig. 1C). UGT2B15 and UGT2B17 are the 2 major enzymes that are responsible for androgen glucuronidation in prostate cancer (10, 14). The transition from LNCaP to the resistant C4-2 model is accompanied by a profound loss of expression of the glucuronidation machinery (Fig. 1, D and E), which accounts for the apparent switch in metabolic phenotype (Fig. 1A).

Glucuronidation deficiency and the metabolic phenotype that sustains active DHT is experimentally inducible with castration

To determine whether the loss of glucuronidation activity that occurs in the C4-2 model that allows the persistence of free DHT is attributable to the development of castration-resistance, LNCaP xenografts were grown in eugonadal or castrated mice and xenograft tissues were interrogated for DHT glucuronidation activity (Fig. 2A). A subset of these tumors lose the capability of rapidly inactivating DHT, which otherwise consistently occurs with LNCaP xenografts grown in eugonadal mice. Expression of UGT2B15 protein was assessed and is lost in tumors with metabolic phenotypes that permit high free DHT compared with those that restrict DHT maintenance (Fig. 2B). A tumor subclone was grown from a xenograft selected for the absence of an ability to inactivate free DHT (Fig. 2A) and stably displayed glucuronidation incompetence (Fig. 2C) that is attributable to loss of UGT2B15 and UGT2B17 expression (Fig. 2D). Together, these results suggest that coordinate UGT2B15 and UGT2B17 loss commonly occurs in the transition to CRPC.

Genetic ablation of UGT2B15 and UGT2B17 is sufficient to allow androgen persistence in cells treated directly or with precursors of testosterone and DHT

To determine whether the androgen-glucuronidation incompentence that occurs in the transition from LNCaP to C4-2 cells is attributable to loss of UGT2B15 and UGT2B17, CRISPR-Cas was employed to genetically ablate these two genes, resulting in loss of transcript and protein expression (Fig. 3, A and B). Loss of UGT2B15 and UGT2B17 in LNCaP is sufficient to block glucuronidation and maintain persistence of free testosterone and DHT in media to an extent that is comparable with that which occurs in C4-2 cells (Fig. 3C). UGT2B15 and UGT2B17 loss also enables sustained intracellular free DHT, as assessed with radioactive signal emitted from [3H]DHT (Fig. 3D) and with the DHT concentration assessed by LC tandem-MS (LC-MS/MS; Fig. 3E). In contrast, glucuronidated androgens predominate to a much greater extent in Ctrl compared with UGT2B15 and UGT2B17 knockout cells (Fig. S1). Furthermore, the concentration of intracellular testosterone and DHT is also augmented in cells that are
UGT2B15 and UGT2B17 deficient when treated with DHEA, a major adrenal precursor steroid that is converted to more potent androgens by steroidogenic enzymes (Fig. 3F).

**Absence of DHT inactivation machinery spurs sustained AR signaling, cell proliferation, and more rapid development of CRPC**

Castration-resistant growth of C4-2 cells is generally thought to be androgen independent (15–18). We sought to determine whether the loss of androgen-glucuronidation activity in this model allows for exaggerated and more sustainable responses to a single treatment with a low concentration of DHT that would provide an alternative explanation to androgen-independent mechanisms. C4-2 cells treated with DHT (10 nM) indeed have sustained induction of DHT-stimulated androgen-responsive gene expression (PSA, FKBP5, and TMPRSS2) compared with the original LNCaP model (Fig. 4A). Genetic elimination of UGT2B15 and UGT2B17 expression from LNCaP recapitulates sustained induction of androgen-regulated transcripts relative to expression in glucuronidation-competent cells (Fig. 4B) and results in augmented responses to direct treatment over a concentration range of DHT (Fig. 4C) as well as the adrenal precursor DHEA (Fig. 4D). Unimpeded exposure to DHEA, testosterone, and DHT, due to loss of glucuronidation activity enables rapid cell proliferation (Fig. 4E) and hastened development of CRPC in vivo (Fig. 4F). Reinstatement of glucuronidation activity reverses prostate cancer accessibility to sustained active DHT (Fig. 4G) and restricts castration-resistant growth (Fig. 4H).

**Tumors permissive for unimpeded androgen exposure are noninvasively distinguishable with PET imaging using \(^{18}F\)-DHT**

We set out to explore alternative approaches that might be feasible for noninvasively distinguishing between androgen-glucuronidation-proficient and androgen-glucuronidation-deficient tumors by exploiting changes in the balance between...
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Figure 4. Genetic loss of UGT2B15 and UGT2B17 promotes sustained androgen-responsive gene expression, cell proliferation, and promotes CRPC growth. A, castration-resistant C4-2 cells have persistent induction of androgen-responsive gene expression with DHT stimulation. Cells were treated with CSS media for 72 h, treated with DHT (10 nM) for the designated incubation times, and expression of PSA, FKBP5, and TMPRSS2 was assessed in triplicate by qPCR and normalized to RPLPO. Compared with WT LNCaP (Ctrl), combined loss of UGT2B15 and UGT2B17 (LNCaP KO) recapitulates sustained androgen-responsive gene expression with (B) DHT (10 nM) treatment over time and in (C) DHT and (D) DHEA concentration-dependent manners. Expression of PSA, FKBP5, and TMPRSS2 was assessed in triplicate by qPCR and normalized to RPLPO and vehicle treatment. In B, expression in KO cells is also normalized to Ctrl cells. Error bars represent the S.D. E, loss of UGT2B15 and UGT2B17 augments androgen-responsive cell proliferation. Cells were treated with vehicle, DHEA (100 nM), testosterone (100 nM), and DHT (10 nM), in triplicate, and counted after treatment for the indicated number of days. F, castration-resistant growth in vivo is hastened in KO (n = 10 mice) compared with Ctrl (n = 10) tumors (p = 0.0002). Three days after subcutaneously injecting cells into NSG mice, mice were surgically orchiectomized and implanted with DHEA pellets to mimic human adrenal physiology. The significance of the difference in progression-free survival between groups, as assessed by tumor volume > 300 mm³, was assessed by log rank test. G, UGT2B15 expression in C4-2 cells reinstates enzymatic DHT glucuronidation. C4-2 cells were stably transfected with control vector (Ctrl) or UGT2B15 (2B15) and expression was assessed by immunoblot. Next, cells were treated with [3H]DHT (10 nM) for 6 h and free and glucuronidated DHT was assessed by HPLC in triplicate. Error bars represent the S.D. H, restoration of DHT glucuronidation activity suppresses castration-resistant xenograft growth. Ctrl and 2B15 cells (n = 7 mice per group) were subcutaneously injected in orchiectomized mice, progression-free survival was assessed as tumor volume > 300 mm³, and the significance of the difference between groups was compared with log rank test; p = 0.0035.

free versus conjugated substrates of glucuronidation. We reasoned that under certain circumstances, nonsteroid substrates of glucuronidation might be advantageous as alternatives to steroid substrates. SCH23390 is a dopamine D1 receptor antagonist that is also a substrate for hepatic glucuronidation (19). Our results demonstrate that [3H]SCH23390 is clearly glucuronidated by prostate cancer cells, although it appears to be a less effective substrate compared with DHT (Fig. 5A). Similar to androgen glucuronidation, SCH23390 glucuronidation is genetically reversible with loss of UGT2B15 and UGT2B17 (Fig. 5B). However, our attempts to image prostate cancer xenografts with [11C]SCH23390 resulted in very little tumor radioactivity uptake. Next, we reasoned that a pulse-chase approach using [3H]DHT would mimic in vivo conditions for PET imaging with [18F]-DHT. [3H]DHT treatment followed by a chase yields higher total radioactivity in glucuronidation-proficient cells compared with glucuronidation-deficient cells that is greatest at earliest time points and diminishes over the washout time (Fig. 5C). Cellular radioactivity residing in the aqueous (glucuronidated) and organic (free) phases of steroid extraction demonstrate that the increase in cellular signal in the glucuronidation-proficient cells is attributable to glucuronidated DHT (Fig. 5D). It should be noted that these data differ from Fig. 3D, which is instead done with continuous DHT treatment, as opposed to a pulse-chase approach. To determine whether the intracellular glucuronidated fraction could be further selectively increased in glucuronidation-competent cells, we performed similar experiments with cyclosporin A (CSA) co-treatment, which is known to inhibit membrane transporters that normally export intracellular substrates and would therefore be expected to increase cellular glucuronidated DHT retention (20). CSA selectively increases radioactivity attributable to [3H]DHT in cells with intact glucuronidation activity (Fig. 5E) and only augments the glucuronidated DHT fraction without any change in intracellular free DHT (Fig. S2). Finally, we sought to determine whether preferential retention is demon-
strable in glucuronidation-competent prostate cancer with 18F-DHT and PET imaging. Matched xenografts that are glucuroni-
dation-competent (Ctrl) and UGT2B15/UGT2B17-defective LNCaP (KO) were grown in mice and treated with CSA imme-
diately prior to administration of 18F-DHT and microPET-CT scanning (Fig. 5F). Control tumors demonstrated higher maximum tracer uptake and more rapid washout compared with KO tumors. There was no difference in AR protein expression between Ctrl and KO cells, supporting a model in which the increase in 18F-DHT uptake in Ctrl tumors occurs independent of AR content (Fig. S3). Together, these results demonstrate that the glucuronidation-deficient metabolic phenotype, which occurs in the transition to castration resistance is detectable with a timed PET imaging approach that assesses the tumor’s functional metabolic status.

Discussion

The past few years has witnessed a dizzying pace of advances in the characterization of prostate cancer genomics (21–23). One of the themes that has emerged from these rich studies is that although there is an incredible level of genetic heterogeneity on one hand, there is also a requirement for a common set of pathways that drive tumor progression and resistance to systemic therapies. Another theme that has developed is the dynamic nature of the repertoire of somatic genetic aberra-
tions, which evolves through response and resistance to systemic treatments (24–26). Long missing have been imaging modalities that capture aspects of the biologic state of the disease in a way that reflects on tumor susceptibility to response or resistance to systemic therapies. Functional imaging modalities are required to capture the dynamic evolution of metastatic
tumors that are spatially and temporally separated. No such imaging modalities are currently employed in standard clinical practice, which are usually instead employed to determine tumor size and/or localization (27, 28).

As currently used, 18F-DHT does not offer any advantages over 18F-deoxyglucose for metastatic prostate cancer localization and neither are routinely used for clinical management (29, 30). PET imaging with 18F-DHT was mainly developed as a high-affinity ligand for localization and expression of AR ligand binding in prostate cancer (31). In our study, we found no increase in AR protein expression in glucuronidation-proficient tumors (Fig. S3), thus excluding the possibility that the increase in 18F-DHT signal that we show in these tumors is due to the known effect and affinity of 18F-DHT for AR (30, 31). In fact, a decrease in free 18F-DHT and AR binding occurring in glucuronidation-proficient tumors probably blunts the detectable difference in PET signal with glucuronidation-defective prostate cancer (Fig. 5). We would anticipate that development of a nonsteroidal glucuronidation substrate may therefore in fact exhibit an accentuated difference in tracer retention between these tumor types. Although we demonstrated that the prostatic glucuronidation machinery that normally physiologically inactivates androgens also conjugates the (nonsteroid) substrate SCH23390, tumor uptake was negligible. Nonetheless, this approach with nonsteroid substrates should be explored further in future studies.

Our approach in some ways mimics the strategy used for the development and use of [18F]fluorodeoxyglucose. Both strategies have in common the use of a tracer that undergoes intracellular modification to a polar and membrane-impermeable metabolite that may be trapped in cells. Although we demonstrate a proof of principle approach for noninvasive detection of a switch in enzymatic steroid inactivation, we acknowledge that our approach may benefit from further optimization.

Finally, we highlight the discovery of a novel metabolic phenotype that enables the sustenance of elevated intratumoral androgens in androgen deprivation therapy resistance, specifically by way of a mechanism downstream of biologically active androgens that blocks intratumoral androgen loss. These studies complement known mechanisms upstream of biologically active androgens that increase synthesis from extragonadal androgen precursor steroids.

**Experimental procedures**

**Cloning and CRISPR cell generations**

UGT2B15 cDNA plasmid was purchased from GE Dharmacon (MHS6278-213245438) and cloned into the lentiviral pCMV vector. LentivCRISPR v2 was used for knockout of UGT2B15 and -2B17. LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid no. 52961). Guide RNA sequence for targeting hUGT2B15 5'-GAACTGAAGACTGTCAGAGAA-GG-3', hUGT2B17 5'-GTTAAGCCATCTGCCAGAAGG-3', and nontargeting control 5'-GTTCCGCTTACATACATAC-TTA-3' were designed, cloned, and virus was produced using the LentiCRISPRv2 protocol (32). To generate the UGT2B15 and UGT2B17 double knockout cell line, LNCaP cells were infected with both viruses simultaneously.

**Cells and culture conditions**

The LNCaP human prostate cancer cell line was purchased from American Type Culture Collection (ATCC) and C4-2 cells were generously provided by Dr. Leland Chung (Cedars-Sinai Medical Center, CA). Cells were maintained in RPMI 1640 (Sigma) with 10% fetal bovine serum (Gemini). All cell lines were incubated in a 5% CO2 humidified incubator and 1% penicillin/streptomycin was added to all culture media. All cell lines were authenticated prior to use and routinely tested for mycoplasma contamination.

**Media and intracellular UGT activity studies**

Radiolabeled steroids were purchase from PerkinElmer. 1 × 10^6 cells were treated in phenol red-free media with charcoal-stripped serum (CSS; Gemini) spiked with 10 μCi of [3H]DHT, [3H]testosterone, or [3H]SCH23390 (PerkinElmer Life Sciences), and 10 nM cold testosterone, 10 nM DHT, or 100 nM SCH23390 (Sigma). Culture media and cell pellets were harvested after the indicated incubation time. For MS analysis, 2 × 10^6 cells were seeded in 15-cm dishes with CSS media 48 h prior to treatment. Media was aspirated and 20 ml of fresh CSS media spiked with DHT (10 nM) or DHEA (100 nM) was added. Cells were collected, centrifuged, weighed, and frozen at −80°C.

As previously described (33, 34), media samples were extracted with 1:1 ethyl acetate:isooctane and cell pellets were re-suspended in 600 μl of PBS and subjected to 3 freeze-thaw cycles before extraction. For analysis of free steroids using HPLC, the organic layer was collected and the solvent evaporated under nitrogen gas in glass tubes. To analyze glucuronidated steroids or SCH23390, the aqueous phase after the first extraction was washed 3 times by ethyl acetate:isooctane, and treated with β-glucuronidase at 37°C for 4 h (for steroids) or overnight (for SCH23390) to remove the glucuronide conjugate. The glucuronidation substrate was extracted again with ethyl acetate:isooctane and analyzed by HPLC. Dried samples were reconstituted in 50% methanol and injected on a Breeze 1525 system equipped with a model 717 plus autoinjector (Waters Corp.) and a Kinetix 100 × 2.1-mm, 2.6 μmol/liter of C18 reverse-phase column (Phenomenex) and methanol:water gradients at 30°C. The column effluent was analyzed using a b-RAM model 3 in-line radioactivity detector (IN/US Systems, Inc.) using Liquiscint scintillation mixture (National Diagnostics). Steroid metabolites were identified using titrated standards of AD, T, DHEA, and DHT (PerkinElmer Life Sciences). For detection using a scintillation counter, samples were extracted with the same volume of ethyl acetate:isooctane. 100 μl of the organic layer or aqueous layer was loaded into scintillation tubes with 3 ml of Liquiscint scintillation mixture and scanned by scintillation counter.

**Transcript and protein expression**

mRNA expression and immunoblot were used. Briefly, total RNA was harvested using the RNeasy kit (Qiagen), and 1 μg of RNA was used in a reverse-transcriptase reaction with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) analysis was performed in triplicate with the following primer sets for PSA (forward: 5'-GCATGGGATGGGAATGATTAGGAAGG-3'; reverse: 5'-CATCAAATCTGAGGTTGTCTGGA-
rabbit anti-UGT2B15 (Abcam), rabbit anti-AR (Sigma), and Protein was resolved by 10% SDS-PAGE and incubated with a treatment.

genes, cells were starved with CSS media 48 h prior to androgen cells (for steroid treated cells). To assess androgen-regulated RPLP0 mRNA was determined by normalizing the sample values to the thermocycling reaction in an ABI-7500 Real-Time PCR SYBR Green Supermix with ROX kit (Bio-Rad) was used for reverse: 5′-CACATTCCCCGGATATGAA-3′; reverse: 5′-AAGGCTAGCTGAATTGACCAA-3′) and the housekeeping gene encoding large ribosomal protein P0 (RAPLP0) (forward: 5′-CGAGGGCAACTGGGAAAAC-3′; reverse: 5′-CACATTCGCCCGATAGTA-3′). The iTaq Fast SYBR Green Supermix with ROX kit (Bio-Rad) was used for the thermocycling reaction in an ABI-7500 Real-Time PCR machine (Applied Biosystems). Accurate quantification of each mRNA was determined by normalizing the sample values to RPLP0 and to control cells (for knockout) or to vehicle-treated cells (for steroid treated cells). To assess androgen-regulated genes, cells were starved with CSS media 48 h prior to androgen treatment.

For Western blot analysis, cells were lysed using 10% SDS. Protein was resolved by 10% SDS-PAGE and incubated with a rabbit anti-UGT2B15 (Abcam), rabbit anti-AR (Sigma), and mouse anti-β-actin (Sigma) antibodies.

**In vitro cell growth assays**

Control and KO cells were starved with CSS media 48 h prior to splitting. 2 × 10⁴ cells were seeded in each well in 12-well plate with 2 ml of CSS media. Ethanol, 100 nM DHEA, 100 nM testosterone, or 10 nM DHT were added at day 0. Cells were grown in triplicate in each group and were counted at the indicated number of days.

**Mouse xenograft studies**

All mouse studies were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic Lerner Research Institute. All NSG male mice (6 – 8 weeks old) were purchased from the Jackson Laboratory and the number of mice used in this study was based on previously published mouse xenograft studies that determined effects of steroid pathway inhibition/augmentation on xenograft growth (33, 35, 36). To mimic human adrenal DHEA production in patients with CRPC, mice were surgically orchietomized and 5 mg, 90-day, sustained-release DHEA pellets (Coy) were implanted subcutaneously.

For evaluation of the role of UGT2B15/17 in tumor progression, 10⁷ vector control or UGT2B15/17 KO cells were subcutaneously injected with 50% Matrigel (BD Biosciences) into 10 eugonadal, castrated, or castrated plus DHEA pellet mice. Xenografts were measured twice a week after tumors became palpable. Tumor progression curves were generated by Prism and the statistical significance were analyzed by log-rank (Mantel-Cox) test.

For testing the effect of UGT activity reintroduction, 1 × 10⁶ UGT2B15- or vector-expressing C4-2 cells were subcutaneously injected into 7 eugonadal or castrated mice with 50% Matrigel. Tumor progression curves were generated by Prism and the statistical significance was analyzed by log-rank (Mantel-Cox) test.

**Xenograft UGT activity assays and cell line subculture**

After removal of necrotic regions, xenograft tumor tissues (0.03–0.04 g each, 3 pieces from each tumor under castration selection, or 1 piece from each eugonadal LNCaP or C4-2 tumor) were weighed and carefully minced in 1.5 ml of CSS media using scissors in a 12-well plate under sterile conditions. Each sample was incubated in CSS media spiked with 10 μCi of [3H]DHT and 10 nm cold DHT. Aliquots of media were obtained after 4 h of treatment and frozen in −20 °C for further analysis. To subculture cell lines from xenografts, 1/10 volume of media with minced tissues were transferred to another a 12-well plate with 1 ml of RPMI 1640 and 10% fetal bovine serum.

**Liquid chromatography-tandem MS analysis of steroid content**

_Extraction protocol_—Cell pellets were thawed from storage at −80 °C and subjected to 5 freeze-thaw cycles, after which each cell pellet sample was added to 200 μl of media and transferred into a glass tube. Samples were spiked with 10 μl of internal standard (50 ng/ml of [2,3,4-13C₃]androstenedione; AD-13C₃; Sigma), and briefly vortexed. After addition of 2 ml of methyl tert-butyl ether (Acros), the glass tube was vortexed for 5 min using a multivortex vortexer (Fisher) and centrifuged for 5 min at 2500 rpm at 4 °C. Samples were then placed on dry ice for 10 min, and the top organic fraction with extracted steroid was collected in a new glass tube. The collected organic layer was evaporated under nitrogen gas at 40 °C and then reconstituted in 110 μl of 50% methanol:water (v/v) and briefly vortexed again. The reconstituted samples were transferred into 1.5-ml microcentrifuge tubes, centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatants were collected for mass spectrometry injections in HPLC vials.

_Instrumentation and data analysis_—The extracted steroids from media or cell pellet samples were quantified using LC tandem MS (LC-MS/MS). The LC-MS/MS system consists of an ultra-pressure LC system (UPLC; Shimadzu Corporation, Japan), comprised of two LC-30AD pumps, a DGU-20A5R vacuum degasser, a CTO-30A column oven, SIL-30AC autosampler, and a system controller CBM-20A and coupled with an API Qtrap 5500 mass spectrometer (AB Sciex, Framingham, MA). Briefly, steroid extract was injected onto the Shimadzu UPLC system, and the steroids were separated through a C18 column (Zorbax Eclipse Plus C18 column 150 × 2.1 mm, 3.5 μm, Agilent, Santa Clara, CA) using a gradient starting from 20% solvent B (acetonitrile/methanol 90/10, v/v) containing 0.2% formic acid) over 4 min and then to 75% solvent B over 10 min, followed by 95% solvent B for 3 min. The steroids were quantified on a Qtrap 5500 mass spectrometer using ESI in positive ion mode and multiple reaction monitoring using characteristic parent → daughter ion transitions for the specific molecular species monitored. [2,3,4-13C₃]Androstenedione was used as an internal standard for calibration of steroids in each sample. Data acquisition and processing were performed using MultiQuant (version 3.0.1) from AB Sciex. The peak area ratio of the analyte over the internal standard was used for quantification purposes. Each sample run included calibration curves with standards for data quantification using the analyte/
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internal standard peak area ratio. Steroid metabolite concentrations in cell pellet samples were quantified by LC/MS/MS analysis and normalized to cell pellet sample mass. Experiments were done in biological triplicate and the mean ± S.D. were calculated.

**Pulse-chase assays**

1 × 10⁶ control and KO LNCaP cells were seeded in poly-DLornithine (Sigma)-coated plates with CSS media for 24 h prior to initiation of treatment. For the pulse step, culture media was aspirated and cells were treated with CSS media spiked with 50 μCi of [3H]DHT and 10 nM cold DHT for 5 min. Then the media was carefully removed and cells were washed once with CSS media with 10 nM cold DHT. For the chase step, cells were treated with CSS media with 10 nM cold DHT and then cell pellets were collect at the indicated time points. Radioactivity was determined by a scintillation counter.

**In vivo PET-CT image and analysis**

Mice were injected subcutaneously in each flank with 1 × 10⁷ control and 7 × 10⁶ KO cells (to compensate for more rapid growth of KO cells) mixed with 50% Matrigel. Mice were cas- trated 1 week before imaging. 0.6 mg of cyclosporin A dissolved in 50 μl of DMSO was injected in tail vein 45–60 min before scanning. Mice were anesthetized (2% isoflurane) and injected with CSS media with 10 nM cold DHT for 5 min. Then the media was carefully removed and cells were washed once with CSS media with 10 nM cold DHT. For the chase step, cells were treated with CSS media with 10 nM cold DHT and then cell pellets were collect at the indicated time points. Radioactivity was determined by a scintillation counter.

**Statistical analysis**

Numerical data were presented as mean ± S.E. Tumor progression curves were generated by Prism and the statistical signif- icance was analyzed by log-rank (Mantel-Cox) test.

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**Author contributions**—Z. Z. and N. S. conceptualization; Z. Z., Y.-M. C., O. S., V. K., M. P. B., J. L., H.-K. K., Z. Li, M. P., F. D., and Z. Lee investigation; Z. Z. visualization; Z. Z. and Y.-M. C. methodology; Z. Z., Z. Lee, and N. S. writing-original draft; Z. Z., Y.-M. C., V. K., F. D., Z. Lee, and N. S. writing-review and editing; V. K. data curation; Z. Lee and N. S. formal analysis; N. S. resources; N. S. project administration.

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