Androgen-dependent repression of ERRγ reprograms metabolism in prostate cancer

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Abstract

How androgen signaling contributes to the oncometabolic state of prostate cancer (PCa) remains unclear. Here we show how the estrogen-related receptor γ (ERRγ) negatively controls mitochondrial respiration in PCa cells. Sustained treatment of PCa cells with androgens increased the activity of several metabolic pathways, including aerobic glycolysis, mitochondrial respiration and lipid synthesis. An analysis of the intersection of gene expression, binding events, and motif analyses after androgen exposure identified a metabolic gene expression signature associated with the action of ERRγ. This metabolic state paralleled the loss of ERRγ expression. It occurred in both androgen-dependent and castration-resistant PCa and was associated with cell proliferation. Clinically we observed an inverse relationship between ERRγ expression and disease severity. These results illuminate a mechanism in which androgen-dependent repression of ERRγ reprograms PCa cell metabolism to favor mitochondrial activity and cell proliferation. Further, they rationalize strategies to reactivate ERRγ signaling as a generalized therapeutic approach to manage PCa.

Introduction

Prostate cancer (PCa) is one of the most common cancers in men and the central role of androgens in the development and progression of this disease is well established. Most PCa will respond favorably to androgen-deprivation therapy (ADT) initially (1), but will later evolve into castration-resistant prostate cancer (CRPC), for which only palliative treatments are available. Several mechanisms are involved in the development of castration resistance, including hyper-activation of the androgen signaling pathway via gene amplification, mutation and alternative splicing of the AR (2). Identifying downstream effectors of the androgen receptor (AR) would lead to a better understanding of the course of PCa and, most importantly, identify novel therapeutic targets.

The prostate gland produces high levels of citrate due to a blunted tricarboxylic acid (TCA) cycle inhibited by high levels of zinc (3). Through the process of malignant transformation, citrate accumulation ceases, which is thought to be the consequence of restored mitochondrial function and increased lipid synthesis from citrate production (3). In PCa cells, androgens have been shown to modulate metabolic gene networks, resulting in the induction of glucose consumption and lactate production (4,5). Moreover, long-term androgen treatment leads to a reprogramming of energy metabolism that increases mitochondrial biogenesis and activity (6). However, mapping genome-wide AR binding sites using chromatin-immunoprecipitation coupled with deep sequencing (ChIP-seq) revealed that metabolic genes are under-represented in AR direct target genes (4,5). Therefore, the androgen-signaling pathway likely requires modulation of effectors downstream of AR to promote metabolic gene expression in PCa.

Estrogen-related receptors α and γ (ERRα and γ) are orphan members of the nuclear receptor superfamily (7). The ERRs are now considered master regulators of energy metabolism (8,9), controlling the vast majority of nuclear encoded mitochondrial genes (10-12). The ERR transcriptional pathway has also been extensively linked with the cancer phenotype (13,14). In breast cancer, ERRα and γ appear to play opposite roles in disease progression (15). ERRα expression correlates with more aggressive tumors while the presence of ERRγ correlates with better outcome and is associated with oxidative metabolism (15-19). In prostate cancer, ERRα expression is also considered as a marker of poor survival, as its expression is higher in cancerous lesions when compared to benign prostate hyperplasia (20). Conversely, there was a trend towards lower survival of patients with low ERRγ expression in a limited immunohistochemistry study (21). Given the high potential for druggability of the ERRs and their prominent role in cell metabolism, it is key to define their respective role in the context of PCa.

In this study, we show that activated AR directly inhibits the expression of ERRγ and that loss of its expression contributes to an androgen-dependent metabolic reprogramming in PCa. Unexpectedly, ERRγ acts as a repressor of oxidative metabolism in PCa cells whereby AR-mediated ERRγ inhibition leads to a de-repression of mitochondrial activity, thus, favoring an oncometabolic state associated with proliferation. The biological significance of these findings is supported by a strong inverse relationship between ERRγ expression and PCa aggressiveness in several independent clinical studies.

Materials and Methods

Cell culture

LNCaP, LAPC4, 22rv1, PC3 and BT-474 cells were originally obtained from ATCC. All cells were kept in culture for no more than 3 months after resuscitation and were re-authenticated using the ATCC cell line authentication service upon completion of the study (July 2016). For treatments with R1881 (10 nM) (Steraloids) or enzalutamide (25 μM) (ApexBio), cells were first deprived from androgens in media with 2% charcoal stripped serum (CSS) for 48 hours, with media changed every 48 hours. For siRNA transfections, cells were trypsinized, seeded in CSS media, and transfected within the following hour with either a non-targeted pool of siRNA (siC), or a pool of siRNA targeting AR, ERRα or ERRγ (SMARTpool™, Dharmacon) with Hiperfect reagent (Qiagen).

RNA extraction and qRT-PCR

RNA was extracted with the RNeasy Mini Kit (Qiagen) and first strand cDNA synthesis was performed with ProtoscriptII reverse transcriptase (New England Biolabs). cDNA expression was quantified by SYBR green based qPCR techniques using the LightCycler480 instrument (Roche). Relative expression was standardized to the expression of housekeeping genes: *PUM1* and *TBP* in LNCaP, and *Rplp0* in mouse tissues. Gene-specific primers can be found in Supplementary Table S1. Two tails Student's t test was used to determine statistical significance.

Protein analyses

Cell lysates were harvested with buffer K or separated into nuclear and cytoplasmic fractions by differential centrifugation as described previously (22). For Western Blots or ChIP, primary antibodies used were: AR (Santa Cruz, sc-816X), ERRα (Abcam, 2131X), ERRγ (gift from Dr. Ronald Evans, Salk Institute), Lamin B1 (Cell Signaling, 12586), YY1 (Santa-Cruz, sc281), and α-Tubulin (Cederlane, CLT-9002).

Animal procedures

Animal procedures were done at the McGill Animal Facility on 12-hour day/night cycle with water and food *ad libidum*. Mice were either castrated or received a sham surgery and sacrificed 7 days after surgery by cervical dislocation. Local ethic committees have approved all animal procedures.

ChIP-qPCR

After steroid deprivation, cells were treated for 16 hours with R1881 for AR ChIP-qPCR or 96 hours for ERRα or ERRγ ChIP-qPCR. ChIPs were performed as described (23) using Dynabeads® (Life Technologies™). Enriched DNA was purified using a Qiagen PCR purification kit and analyzed by qPCR using LightCycler 480®. Non-targeted rabbit IgG ChIP was used as a control of antibody non-specific binding and 2-3 negative regions were used for ChIP normalization between samples. Gene-specific primers can be found in Supplementary Table S2. ChIP-seq data for AR in LNCaP cells were from publicly available published data, identifying 2,473 unique genes with at least one AR binding sites ± 20kb of the TSS of known genes when using the 11,053 AR

peaks identified by Massie et al. (6) and after processing them with the LiftOver tool of the UCSC Genome Bioinformatics toolbox from hg18 to hg19.

Metabolic analyses

Levels of metabolites consumed and produced by PCa cells in the culture media were measured with the BioProfile Analyzer (Nova Biomedical). Cells were treated for a total of 4 days, with new treatment added after 48 hours.

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using Seahorse XF24 and XFe96 (Seahorse Bioscience). 48 hours after siRNA transfection, cells were treated with vehicle or R1881 for 48 hours, then trypsinized and seeded in Seahorse plates. After 24 hours, RPMI was replaced by Seahorse assay media and cells were incubated in a CO_2-free incubator at 37ºC for 1 hour prior to metabolic analysis. Following ECAR and OCR analyses, cells were trypsinized for cell count and data normalization.

TCA cycle intermediates were measured at the Metabolomics Core Facility of the Goodman Cancer Research Centre using a standard protocol (24). For triglyceride quantification, lipids were then quantified using the Abcam triglyceride quantification assay kit (colorimetric). Metabolic results were normalized for cell number.

Microarrays

RNA was purified and sent for microarray analysis at Génome Québec and McGill University Genome Centre (Illumina HumanHT-12 Expression Beachip v4). FlexArray software was using for microarray data normalization. Ingenuity™ Pathway Analysis

(IPA), using probes with $p<0.05$, and Gene Set Enrichment Analysis (GSEA), using *Hallmarks* pathways, were used to analyze pathway enrichment. HOMER was used for motifs analysis (25). Data were processed with Gene Cluster 3.0, using the hierarchical linkage method followed by manual curating on log-transformed data, and clusters were visualized using JavaTreeView (26). Microarray data are available in the Gene Expression Omnibus (GEO; GSE86781).

Clinical data and statistical analyses

For ERRγ expression data in clinically localized prostate cancer treated by radical prostatectomy and for association with recurrence, we used data (GEO Series GSE25136) from Sun and Goodison, (27) and provisional TCGA data from February 3, 2016 (TCGA Research Network: http//cancergenome.nih.gov/). For analysis of primary prostate tumors compared to metastatic tumors, microarray data described previously were reanalyzed (GDS2545 and GSE3325)(28-30). For Kaplan-Meier, log-rank test, and Cox regression analysis, data from Taylor et al. (GSE21032) (31) were analyzed through the Project Betastasis webpage (www.betastasis.com) and using XLSTAT.

Microarray data from Sun et al. (32) were analyzed for ERRα and ERRγ expression in human LuCaP35 xenografts. Expression data form the mouse model with loss of *Pten* with or without *K-ras* activation were taken from GSE34839 (33).

Results

Androgens reprogram PCa cell metabolism

The androgen-signaling pathway has been shown to regulate several metabolic pathways including both aerobic glycolysis and mitochondrial respiration (4,6,34). Accordingly, following sustained treatment of the synthetic androgen R1881, we observed an increase of about 400% in glucose uptake (Fig. 1A), as well as a significant increase in lactate production (Fig. 1B) in LNCaP-treated cells compared to controls. The extracellular acidification rate (ECAR), an indicator of glycolysis, was induced by more than 2-fold following androgen treatment (Fig. 1C). These results indicate that androgens are a driving force that increase glucose usage, consistent with the reported regulation of glycolytic genes such as hexokinases I and II (*HK1* and *HK2*) by the AR (4). Androgens also stimulated mitochondrial respiration as demonstrated by the increase in oxygen consumption rate (OCR) of nearly 200% following 3-days stimulation by R1881 (Fig. 1D and E). Androgens also promoted lipid accumulation (Supplementary Fig. S1A). R1881 treatment of a second human androgen-dependent PCa cell line, LAPC4, also induced glucose consumption (Fig. 1F), glycolysis (Fig. 1G and H), and OCR (Fig. 1I and J) in a similar manner. These data indicate that sustained androgen stimulation promotes global cellular energy metabolism.

Downstream effectors of the AR transcriptional pathway are required for androgen-mediated metabolic reprogramming

To study how androgens modulate metabolism, we investigated global changes in transcriptional programs following 72 hours treatment with R1881 in LNCaP cells. As

expected, the most significantly enriched gene signature in treated cells was the "androgen response" (Supplementary Fig. S1B). Several gene signatures associated with cell metabolism were also significantly enriched in R1881-treated cells compared to controls. Gene signatures related to glycolysis (Fig. 2A), oxidative phosphorylation (Fig. 2B) and fatty acid metabolism (Fig. 2C) were all increased following sustained androgen stimulation. When considering genes with at least one AR peak ± 20kb of their transcriptional start site (TSS), out of the 139 genes included in the core enrichment of these three androgen modulated metabolic pathways, only 18 and 11 (13% and 8%) were direct AR targets according to previously published ChIP-seq data generated from LNCaP cells and CRPC tumors (Fig. 2D and Supplementary Fig. S1C) (4,5). Even when considering peaks over 1 X 10^6 bp away from TSS in LNCaP cells AR ChIP-seq data, only 36 out of the 139 metabolic core genes had an identifiable AR peak, indicating that the vast majority of these genes are indirectly regulated by R1881 treatment. We thus hypothesized that other transcription factors may be required downstream of the AR to sustain the metabolic program induced by androgens. Therefore, we performed DNA motif search in the promoter region of the 121 metabolic core genes not directly bound by AR in LNCaP cells (Fig. 2E). Only two known DNA motifs were significantly enriched in these promoters: the ERR response element (ERRE; p=1x10^{-5}) and the nuclear respiratory factor 1 DNA binding motif (NRF1; p=1x10^{-2}). Similar results were also obtained using AR ChIP-seq data generated from CRPC tumors (Supplementary Fig. S1D). Both motifs are associated with transcription factors known to regulate nuclear-encoded mitochondrial genes.

ESRRG is a direct AR target

As both ERRα and γ isoforms recognize the same ERRE motif, we first determined whether androgens could regulate expression of one or both ERR isoforms. ERRα expression was not affected by androgens, even after a 3-day treatment (Fig. 3A and Supplementary Fig. S2A and S2B). In contrast, ERRγ expression was significantly downregulated following exposure to R1881 at both the mRNA and protein levels (Fig. 3A). This decrease was stable over time, lasting at least 4 days (Supplementary Fig. S2C). Conversely, decreased androgen availability through serum starvation led to an induction of ERRγ expression in LNCaP cells (Supplementary Fig. S2D). Androgen deprivation *in vivo* as a result of castration also led to a significant increase of ERRγ in human prostate cancer xenografts of LuCaP35 (32), while ERRα expression again remained unchanged (Fig. 3B). ERRγ was also induced by castration in the mouse ventral and dorsolateral prostate lobes (Fig. 3C), while being less responsive in the anterior lobe (Supplementary Fig. S2E). We did not detect significant change in *Esrra* expression in various mouse prostate lobes (Supplementary Fig. S2E and S2F). Taken together, these data indicate that androgens regulate the expression of ERRγ *in vitro* and *in vivo,* in both the normal prostate and PCa cells.

Our re-analysis of AR ChIP-seq data identified three AR binding sites within the *ESRRG* locus (Fig. 3D). R1881 treatment led to a significant recruitment of AR at all three binding sites in LNCaP cells as measured by ChIP-qPCR (Fig. 3D and Supplementary Fig. S2G). This observation was further validated in two other AR-positive PCa cell lines, 22rv1 and LAPC4 (Fig. 3D and Supplementary Fig. S2H and S2I). *ESRRG* is also a strong AR direct target in CRPC tumors (5). AR knockdown using

siRNAs significantly impaired ERRγ repression by androgens both at the mRNA and protein levels in LNCaP cells (Fig. 3E and Supplementary Fig. S2J). Treatment of LNCaP cells with the AR antagonist enzalutamide (MDV3100) led to a significant increase in ERRγ expression, and completely abolished the androgen-mediated repression of ERRγ (Fig. 3F).

ERRγ regulates the expression of metabolic genes in PCa cells

We next examined the possibility that modulation of the expression of ERRγ, and consequently of its downstream targets, could function as an effector of the AR in the reprogramming of cellular metabolism in PCa. We first performed standard ChIP-qPCR experiments to validate that ERRγ, as previously observed in other cell types (17,35-37), targets known ERR-modulated metabolic genes in PCa cells. As shown in Fig. 4A and Supplemental Fig. S3A, ERRγ is strongly bound to the promoter of several metabolic genes in the absence of androgen. Notably, several of these genes such as *ATP5B* as well as *FH*, *IDH3A*, *SDHB* and *SDHD* were previously identified in the metabolic signatures associated with sustained R1881 treatment (Fig. 2). ERRγ binding to these metabolic target genes was significantly decreased upon R1881 exposure, which is consistent with a reduction in ERRγ protein levels (Fig. 3A and 4A). We observed between 2- to 4-fold decreases in ERRγ binding following androgen treatment on all targets (Fig. 4A and Supplementary Fig. S3A). We next tested whether an increase in ERRα binding, which recognizes the same response element present in these genes, could compensate for the decreased ERRγ binding to these regions. However, ERRα binding was unchanged following R1881 (Supplementary Fig. S3B).

To understand the functional consequence of ERRγ repression on cell metabolism, we conducted microarray experiments in LNCaP cells following siRNA-mediated ERRγ repression, with or without R1881 treatment (Fig. 4B and Supplementary Fig. S3C). In the absence of androgens, ERRγ knockdown significantly altered the expression of 3,726 genes. IPA analyses revealed that genes enriched in our microarray analysis following ERRγ knockdown in the absence of androgens displayed enrichment for metabolic pathways such as fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS), but also folate metabolism, a biochemical pathway recently identified as being regulated by ERRα (Fig. 4C) (38). Gene set enrichment analysis (GSEA) also revealed that metabolic pathways were strongly affected. Notably, the TCA cycle pathway was enriched following ERRγ knockdown, with the expression of genes such as *FH* and *SDHB* being increased significantly (Fig. 4D and E, Supplementary Fig. S3D). Importantly, the effect on transcriptional regulation of these genes by inhibition of ERRγ was similar to that of treatment with R1881. Genes associated with glycolysis were also enriched in cells with ERRγ knockdown (Supplementary Fig. S3E).

To further understand which ERRγ targets were associated with the androgen response, we intersected mRNA targets that were altered by either repression of ERRγ or androgen treatment; the latter also exhibiting decreased ERRγ levels and activity. By clustering the probes correlating following R1881 treatment or ERRγ knockdown, we identified 886 repressed and 526 activated genes (Fig. 4F). Interestingly, genes induced following ERRγ knockdown or androgen treatment were associated with cell metabolism and included pathways related to cholesterol and acetyl-CoA synthesis, TCA cycle, and glycolysis (Fig. 4F). Accordingly, both ERRγ knockdown or R1881 treatment affected

TCA cycle genes such as *FH* and *SDHB* by inducing their expression (Fig. 4D). Taken together, these results suggest that androgens modulate metabolic genes in part through direct repression of ERRγ.

Loss of ERRγ promotes oxidative metabolism in PCa cells

To gain more insight into the function of ERRγ on cell metabolism, we next focused on its metabolic role under steroid deprivation, when it is expressed at high levels. Expression data in PCa cells showed increased expression of genes related to mitochondrial activity and TCA cycle following ERRγ knockdown (Fig. 4) suggesting that, in the context of PCa cells, ERRγ represses mitochondrial activity. To validate this hypothesis, both ECAR and OCR were measured in LNCaP cells lacking ERRγ. Depletion of ERRγ significantly decreased ECAR and increased OCR resulting in an increase in the OCR/ECAR ratio (Fig. 5A). Knockdown of ERRγ using siRNA also increased OCR in LAPC4 cells (Supplementary Fig. S4A).

To further validate our results, we performed a stable infection of LNCaP cells with ERRγ overexpression or control vector (Supplementary Fig. S4B and S4C). ERRγ overexpressing cells exhibited the opposite metabolic profile compared to cells exposed to siERRγ, with a significant increase in ECAR paired with a significant decrease in OCR and the OCR/ECAR ratio (Fig. 5A). Consistent with these results, levels of TCA cycle intermediates such as citrate and α-ketoglutarate were significantly elevated upon ERRγ knockdown while they were significantly reduced upon ERRγ overexpression (Fig. 5B). These results indicate that ERRγ is a repressor of oxidative metabolism in PCa cells.

To further understand the interplay between ERRγ, androgens, and PCa metabolism, we investigated the impact of ERRγ modulation following androgen stimulation. Treatment of LNCaP cells with 72 hours treatment with R1881 concomitant with ERRγ knockdown did not further increase OCR when compared to control cells with androgen (Supplementary Fig. S4D), consistent with a strong repression of ERRγ by R1881 (Fig. 3 and 4). Repression of ERRγ with two distinct shRNAs gave similar results and significantly increased OCR in LNCaP cells in absence of androgens, mimicking R1881 treatment effect (Supplementary Fig. S4E and S4F). In cells stably transfected with the control vector of our overexpression system, R1881 repressed *ESRRG* expression as it did in parental cells (Supplemental Fig. S4B and S4C). Importantly, ERRγ rescue (overexpression; Supplemental Fig. S4C) significantly decreased the respiratory capacity of LNCaP cells compared to control cells, both in absence and presence of R1881 (Fig. 5C). ECAR, strongly induced by R1881, was also impaired by ERRγ overexpression, but at a lesser extent than OCR indicating a dominant role of AR in modulating aerobic glycolysis capacity of PCa cells (Fig. 5D). Overall, overexpression of ERRγ impaired the ability of R1881 to induce global metabolism (Fig. 5D). Finally, we tested the biological consequence of ERRγ modulation in the CRPC cell line PC3. As observed in androgen-dependent cell lines, ERRγ acted as a repressor of mitochondrial respiration as its repression significantly increased OCR (Supplementary Fig. S4G). As an additional control, we show that by lowering the levels of ERRγ in PCa cells exogenously expressing ERRγ the respiratory capacity of these cells was rescued (Fig. 5E and Supplemental Fig. S4H). These results nicely parallel the mRNA levels of ERRγ target

genes, *ACADM* and *SDHB*, whose expression is also rescued in this context (Supplemental Fig. S4I and S4J).

We next investigated the effect of ERRγ knockdown on LNCaP cell proliferation. Knockdown of endogenous ERRγ with siRNA or shRNA significantly increased cell proliferation, both in absence and presence of androgen (Fig. 5F-G). As both androgens and RNAi repressed *ESRRG*, we hypothesized that the additive effect on cell proliferation could be due to a more rapid metabolic reprogramming of PCa cells. Indeed, *ESRRG* expression was reduced more rapidly when combining siERRγ and a 48-hours treatment with R1881 (Supplemental Fig. S5A). Consistent with these results, differences in mRNA expression levels of the ERRγ target gene *ACADM* and of OCR were more pronounced at 48 hours between siC and siERRγ following androgen stimulation (Supplemental Fig. S5A and S5B). On the other hand, overexpression of ERRγ significantly decreased cell number in LNCaP (Fig. 5H) and PC3 cells (Supplementary Fig. S5C). Therefore, ERRγ expression is inversely associated with cell proliferation.

ERRγ expression decreases with PCa progression

We next assessed the relationship between ERRγ mRNA expression levels and PCa aggressiveness by analyzing several publicly available expression data sets. First, we re-analyzed data from a mouse model of PCa with loss of *Pten*, in which *K-ras* was activated to promote a more aggressive phenotype (33). In this context, ERRγ expression was significantly reduced in the more aggressive condition (*Pten* null with *K-ras* activation; Fig. 6A).

In human PCa samples, ERRγ expression was significantly lower in metastasis compared to primary tumors in two independent clinical data sets (Fig. 6B and C). Moreover, we found that recurrent PCa following radical prostatectomy had lower ERRγ expression compared to non-recurrent diseases (Fig. 6D). Importantly, this was also validated using a large dataset from the TCGA (n=429 patients with data on ERRγ expression). Again, recurrent diseases were characterized by significantly lower levels of ERRγ (Fig. 6E). In a third independent cohort, we further validated that low expression of ERRγ was significantly associated with a higher recurrence rate following radical prostatectomy (p=3x10^{-5}; Fig. 6F), while no association was observed for ERRα (Supplementary Fig. S6). In the data from Taylor et al. (31), when considering several clinical parameters such as Gleason score and age at diagnosis, low ERRγ expression levels were not significantly associated with BCR, even though a trend for increased risk was observed, with a risk ratio of 1.70 (Supplemental Table S3). Using backward stepwise Cox regression to determine the best predictive model of recurrence, low ERRγ expression levels was retained and was significantly associated with an increased risk, with a hazard ratio of 2.5 (p=0.033), together with pathological Gleason score and tumor stage (Fig. 6G and Supplemental Table S3).

Discussion

In this study, we have shown that, while androgens strongly influence the metabolic state of PCa cells, differential regulation of the AR downstream effector ERRγ is required to achieve this phenotype. Indeed, intersection of gene expression, binding-events and motif finding analyses following androgen exposure identified a metabolic gene signature

associated with the action of a member of the ERR subfamily of nuclear receptors. We further showed that, by directly and specifically suppressing the expression of the ERRγ isoform, the androgen-signaling pathway favors a metabolic state that is associated with the growth of PCa cells. Unexpectedly, we showed that ERRγ acts as a repressor of mitochondrial respiration in that cellular context. The ERRγ-dependent metabolic state was observed in both androgen-dependent and CRPC cells, and ERRγ expression was found to be inversely associated with disease aggressiveness in several independent clinical datasets. Taken together, our results clearly identify ERRγ both as a biomarker of disease progression and as a potential novel therapeutic target that, if re-activated, could reverse an oncogenic metabolic program enabling PCa progression.

Restoration of mitochondrial respiration is a key step of prostate carcinogenesis that contributes to increase ATP production (3,39). Augmentation of mitochondrial activity is also linked to several biosynthetic pathways that can favor tumor cell proliferation such as one-carbon metabolism for DNA synthesis (40,41). The AR acts as a strong positive modulator of mitochondrial metabolism (Fig. 1 and 2) (6). Accordingly, induction of mitochondrial activity as reported herein is in agreement with prior findings that androgen-mediated metabolic reprogramming is associated with PCa progression (6,42,43).

It has been well demonstrated that ERRα and γ function as integral regulators of cellular energy metabolism (8,15,44). Indeed, the ERRs bind to and regulate most nuclear-encoded mitochondrial genes, as well as genes associated with other metabolic pathways such as glycolysis and lipid synthesis (10,11,22). Despite strong overlapping in their global DNA binding site distribution and functions (35), it is clear that each ERR

isoform also plays independent roles in development, physiology and disease (15,17,36,45-47). In addition, much remains to be learned on how the expression of each receptor isoform is regulated in both normal and cancer cells. Here, we describe a regulatory mechanism that specifically governs ERRγ activity. Activation of the androgen-signaling pathway significantly repressed ERRγ expression in an AR-dependent manner in both normal and prostate cancer cells without affecting ERRα expression. As ERRγ represses these genes, inhibition of ERRγ activity would release these genes from this negative regulation signal and allow them to be re-expressed. Additionally, loss of ERRγ in this context may promote enrichment of ERRα homodimers at metabolic genes instead of ERRα/ERRγ heterodimers, which are thought to be less active (35,48). Such a change in ERRα occupancy would not be easily discernable using standard ChIP-qPCR. Nonetheless, modulation of ERRγ expression by androgens clearly results in the alteration of a gene program highly associated with oxidative energy metabolism, while AR had a dominant effect on aerobic glycolysis. Functional studies identified ERRγ as a repressor of mitochondrial respiration in PCa cells. In contrast, ERRγ has been shown to act as an activator of mitochondrial activity in muscles as well as in breast cancer cells (17,47). The activity of ERRγ is likely dependent on functional interactions with cell-specific transcription factors and co-regulators. Development of selective ERRγ modulators could potentially be envisaged to induce a transition from a repressor to an activator state of the receptor in PCa cells.

In conclusion, we uncovered a mechanism by which AR-dependent repression of ERRγ contributes to the establishment of an oncogenic metabolic phenotype in PCa cells that favors mitochondrial activity and cell proliferation. Although the expression of ERRγ

in the normal prostate is likely to be regulated according to developmental and physiological needs, ERRγ is expected to be constantly repressed under constitutive AR signaling in PCa cells. The biological and pathological significance of these findings is corroborated with the observation that lower expression of ERRγ is associated with increased PCa aggressiveness in a number of pre-clinical and clinical studies (Fig. 6). Thus, ERRγ expression status emerges as a novel biomarker of PCa progression, while its re-activation represents a potential therapeutic avenue for the management of PCa. As ERRγ expression is induced by anti-androgens such as enzalutamide (Fig. 3F), AR silencing in combination with an activator of ERRγ would likely contribute substantial clinical benefits in the treatment of advanced PCa.

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Figure Legends

Figure 1.

Androgens induce a metabolic reprogramming in prostate cancer cells. **A**, Glucose consumption and **B**, lactate secretion in extracellular media of LNCaP cells following treatment with R1881. One representative experiment performed in triplicate is shown (n=4). **C**, extracellular acidification rate (ECAR) and **D**, oxygen consumption rate (OCR) of LNCaP cells treated with R1881. **E**, metabolic phenotype of LNCaP cells treated with R1881. One representative experiment out of 5 independent experiments is shown. **F**, glucose consumption and **G**, lactate secretion in extracellular media of LAPC4 cells following treatment with R1881. One representative experiment performed in triplicate is shown (n=3). **H**, ECAR and **I**, OCR of LAPC4 cells treated with R1881. **J**, metabolic phenotype of LAPC4 cells treated with R1881. One representative experiment out of 3 independent experiments is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to controls or as indicated. For all experiments, values were normalized for cell number following each experiment.

Figure 2.

Sustained androgen treatment induces a metabolic signature associated with the AR and the ERRs. Gene Set Enrichment Analysis of LNCaP cells following treatment with R1881 indicates a significant enrichment of genes associated with glycolysis in **A**, oxidative phosphorylation in **B**, and fatty acid metabolism in **C**, compared to vehicle. Only genes identified as "core genes" are shown in heatmaps. **D**, overlap between genes with at least one AR binding sites ± 20kb their transcriptional start site in ChIP-seq analysis and core metabolic genes induced by R1881 in LNCaP cells. **E**, DNA Motifs enrichment analysis in the promoter of the 121 metabolic genes not bound by AR.

Figure 3.

Androgens regulate ERRγ expression *in vivo* and *in vitro* in healthy and tumor tissues. **A**, relative mRNA expression of ERRα and ERRγ in LNCaP cells following androgens treatment. Vehicle conditions were set at 0. Results are shown as the average ± SEM of 3 independent experiments performed at least in duplicate. Protein expression of ERRα and

ERRγ following treatment with androgens in LNCaP cells; inset. Lamin B1 is shown as a loading control. **B**, ERRγ (*ESRRG*) expression is induced *in vivo* by castration in human prostate cancer xenografts of LuCap35. Data are from publicly available microarrays published by Sun et al. (32). **C**, ERRγ (*Esrrg*) expression is induced by castration *in vivo* in mouse ventral and dorsolateral prostate lobes (n ≥ 5 mice per group). **D**, ChIP-qPCR analysis of AR binding following treatment with R1881 at 3 genomic regions in *ESRRG* in LNCaP, 22rv1 and LAPC4 cells (n=3). Three transcriptional variants of *ESRRG* of different lengths are shown. **E**, Relative mRNA expression of ERRγ with or without RNAi against the AR in LNCaP cells treated with R1881 (left panel). Control cells treated with vehicle were set at 0. Results are shown as the average ± SEM of 3 independent experiments performed in triplicate. Protein levels of AR and ERRγ with or without RNAi interference against the AR in LNCaP cells treated with R1881 (right panel). Tubulin and Lamin B1 are shown as loading controls. **F**, ERRγ relative mRNA repression in LNCaP cells following treatment with R1881, with or without treatment with the anti-androgen MDV3100. Results are shown as the average ± SEM of 3 independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to controls or as indicated.

Figure 4.

ERRγ transcriptional control of metabolic gene programs. **A**, ChIP-qPCR of ERRγ with or without androgens (n=3). **B**, ERRγ repression following siRNA transfection at the protein level. Microarray analyses were performed from LNCaP cells first transfected with siERRγ or siC and then treated for 72 hours with R1881 or vehicle. **C**, most significantly enriched pathways (IPA) in LNCaP cells following ERRγ inhibition by RNAi. A value over 1.3 indicates significance with $p<0.05$. **D**, relative mRNA expression of *SDHB* and *FH* in LNCaP cells transfected with siC or siERRγ and then treated with R1881 or vehicle. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control. Results are shown as the average ± SEM of 3 independent experiments performed in triplicate. **E**, GSEA analysis identifies the TCA cycle as upregulated in cells with ERRγ inhibition under androgen deprivation. F, Cluster analysis of genes modulated by ERRγ knockdown

and with a similar modulation by R1881 treatment (left panel) with their associated pathways using IPA (right panel).

Figure 5.

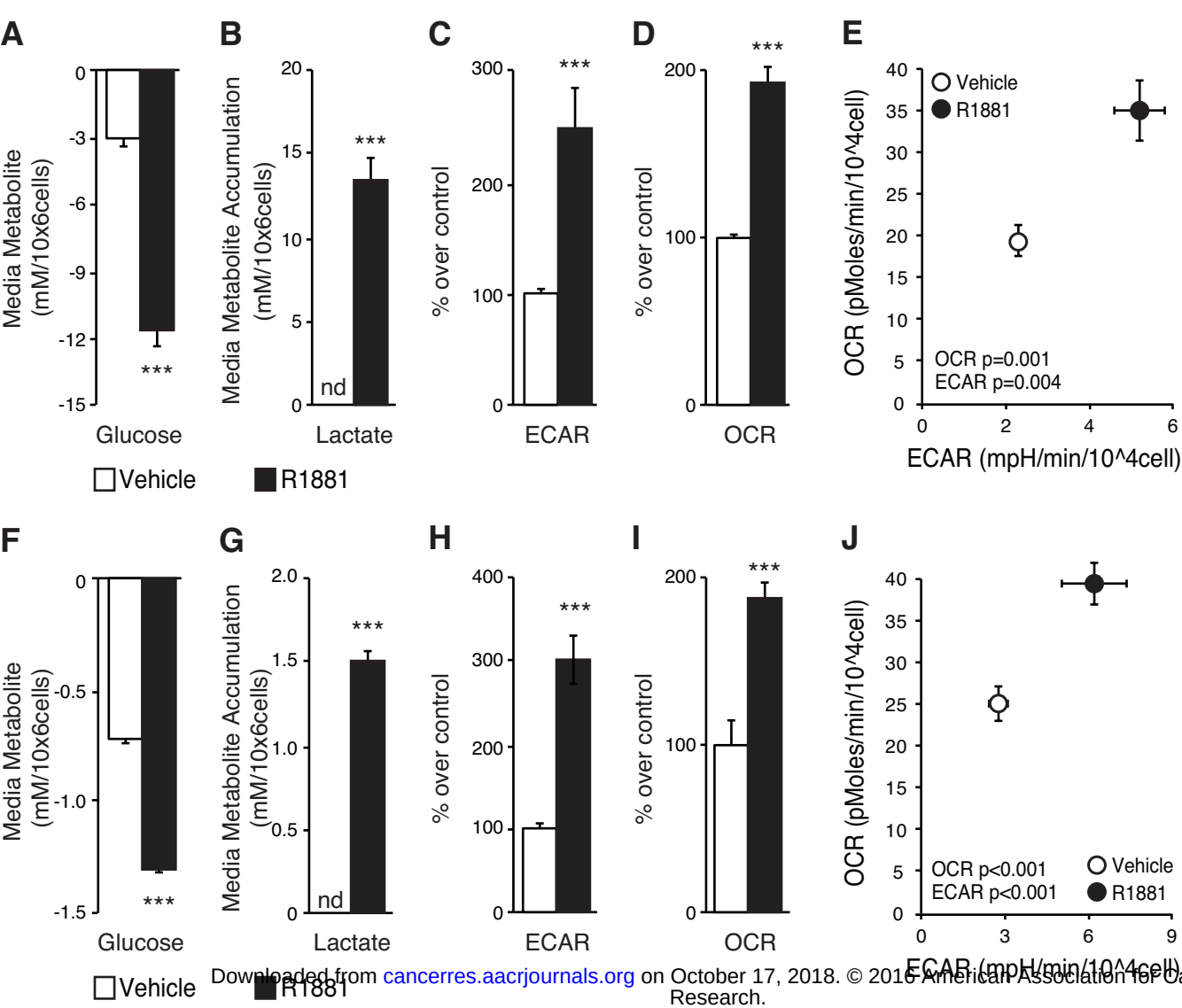
Loss of ERRγ promotes oxidative metabolism and proliferation in prostate cancer cells. **A**, following ERRγ knockdown by siRNA or its overexpression, the extra-cellular acidification rate (ECAR), the oxygen consumption rate (OCR), and the OCR/ECAR ratio were measured in LNCaP cells under steroid deprivation. **B**, levels of TCA cycle intermediates following ERRγ knockdown or overexpression. Log-transformed data are shown normalized to their respective controls. **C**, oxidative capacity and uncoupling respiration of LNCaP cells stably overexpressing ERRγ following 72 hours treatment with R1881. **D**, metabolic organization of control cells or cells overexpressing ERRγ following 72 hours treatment with R1881. **E**, OCR of LNCaP cells with or without siERRγ in absence or presence of ERRγ overexpression following a 72 hours treatment with R1881 or vehicle. **F**, relative cell number of LNCaP cells transfected with siC or siERRγ following a 72 hours treatment with R1881. **G**, relative cell number of LNCaP cells stably expressing shRNA against ERRγ or control (shNTC) following a 72 hours treatment with R1881. **H**, relative cell number of LNCaP cells overexpressing ERRγ or controls following 72 hours treatment with R1881. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For metabolic analyses, values were normalized for cell number following each experiment. C and D are shown as a representative experiment performed with 5 samples per group; E is shown as the average ± SEM of 2 independent experiments (n≥5 samples per group); for other panels, results are shown as the average ± SEM of at least 3 independent experiments performed at least in triplicate.

Figure 6.

ERRγ expression is a biomarker of PCa progression. **A**, *Esrrg* mRNA expression is lower in more aggressive PCa mouse models. Analysis of prostate tumors from mouse lines with prostate-specific Pten loss with or without K-ras activation (33). **B**, relative expression levels of ERRγ (*ESRRG*) in primary (n=65) and metastatic (n=25) tumor samples (28,30). **C**, relative expression levels of ERRγ in clinically localized primary

prostate tumors (n=7) and metastatic tissue pools (n=6) (29). **D**, relative expression levels of ERRγ in non-recurrent (n=40) and recurrent (n=39) PCa tumor samples in GEO profile GSE25136 (27). **E**, relative expression levels of ERRγ in non-recurrent and recurrent PCa tumor samples in the provisional TCGA prostate cancer cohort (n=429, 59 patients had recurrence). **F**, Kaplan-Meier curves of patients with PCa following radical prostatectomy (n=141) from Taylor et al. (31). ERRγ expression is defined as low (lowest 25%) or high (highest 75%). The log-rank test *p* value is shown. **G**, Cox regression following backward selection model of risk variables modulating biochemical recurrence in data from Taylor et al. (31). Low *ESRRG* expression levels is the lowest 25% (highest 75% with a hazard ratio of 1.0).

Audet-Walsh_Fig1



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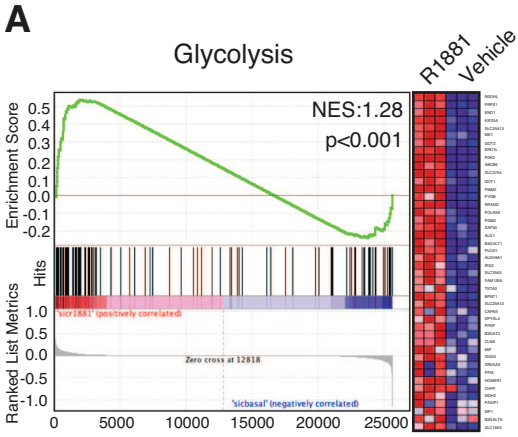

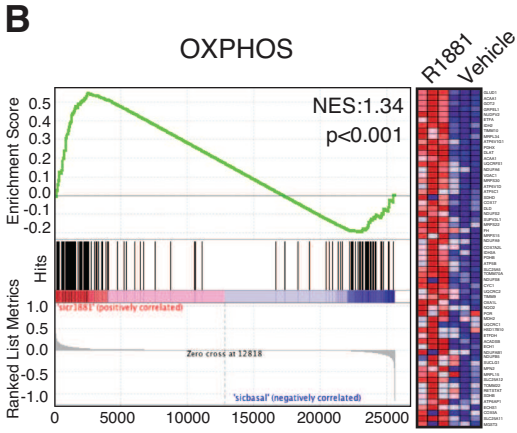

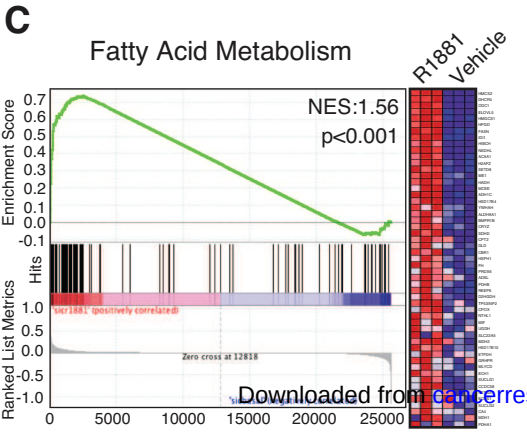

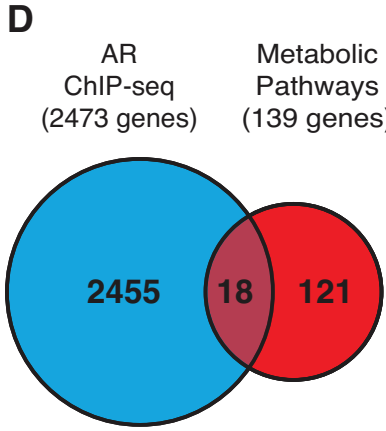

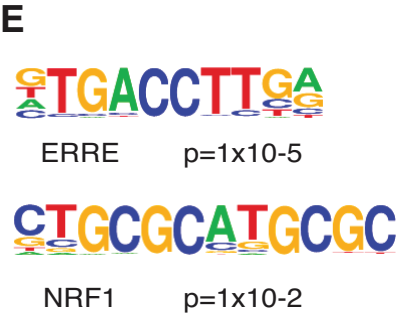

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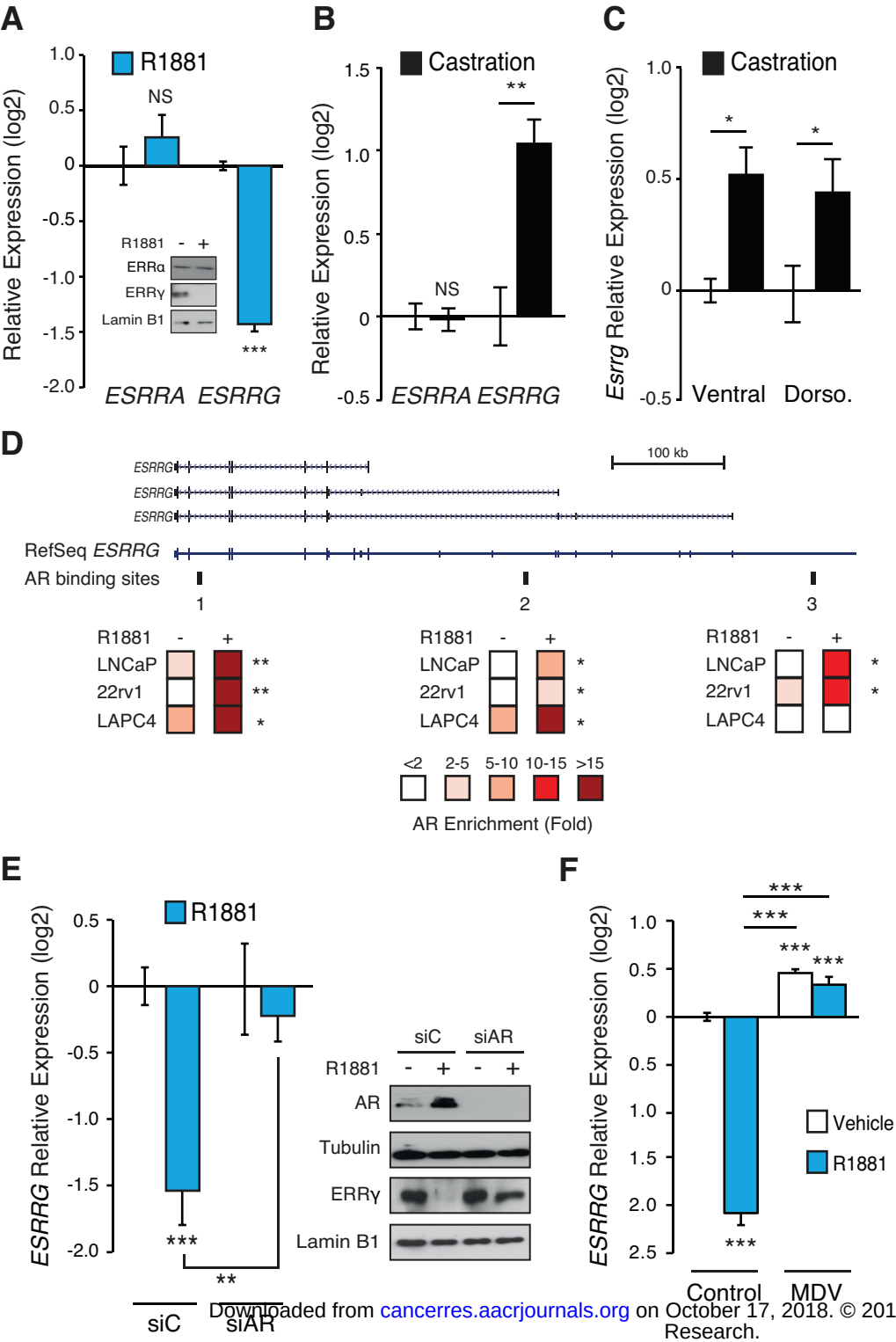

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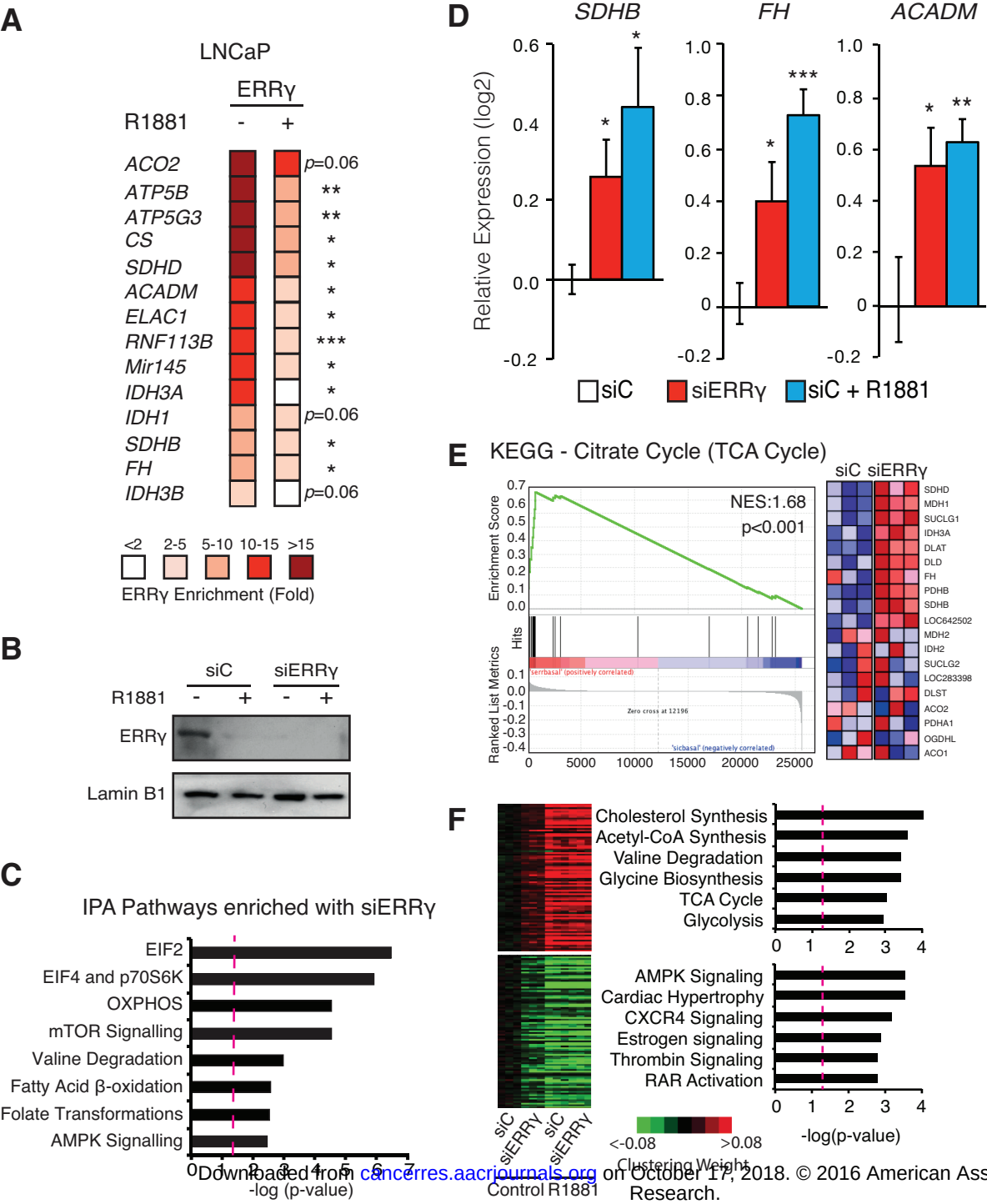

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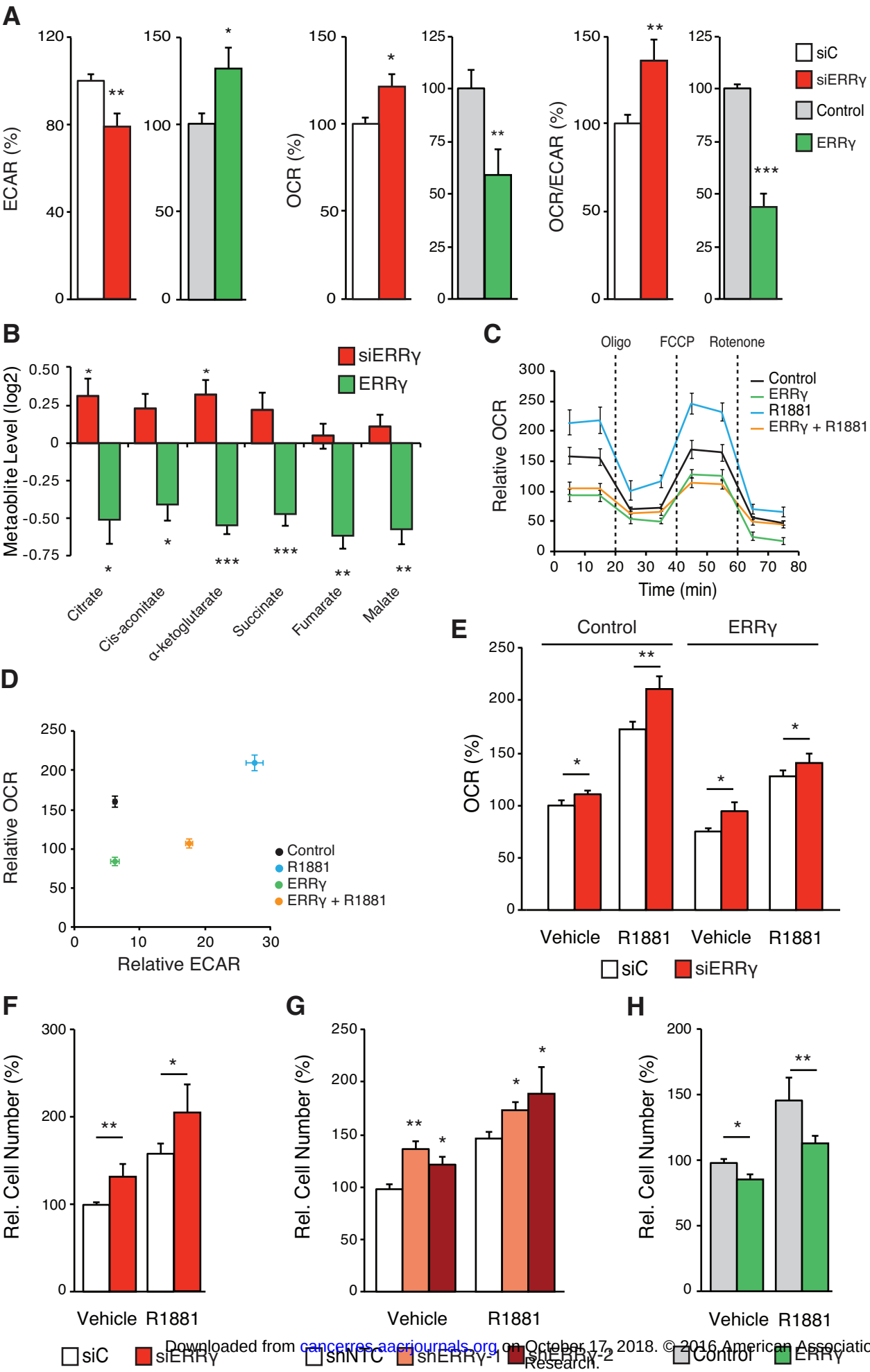

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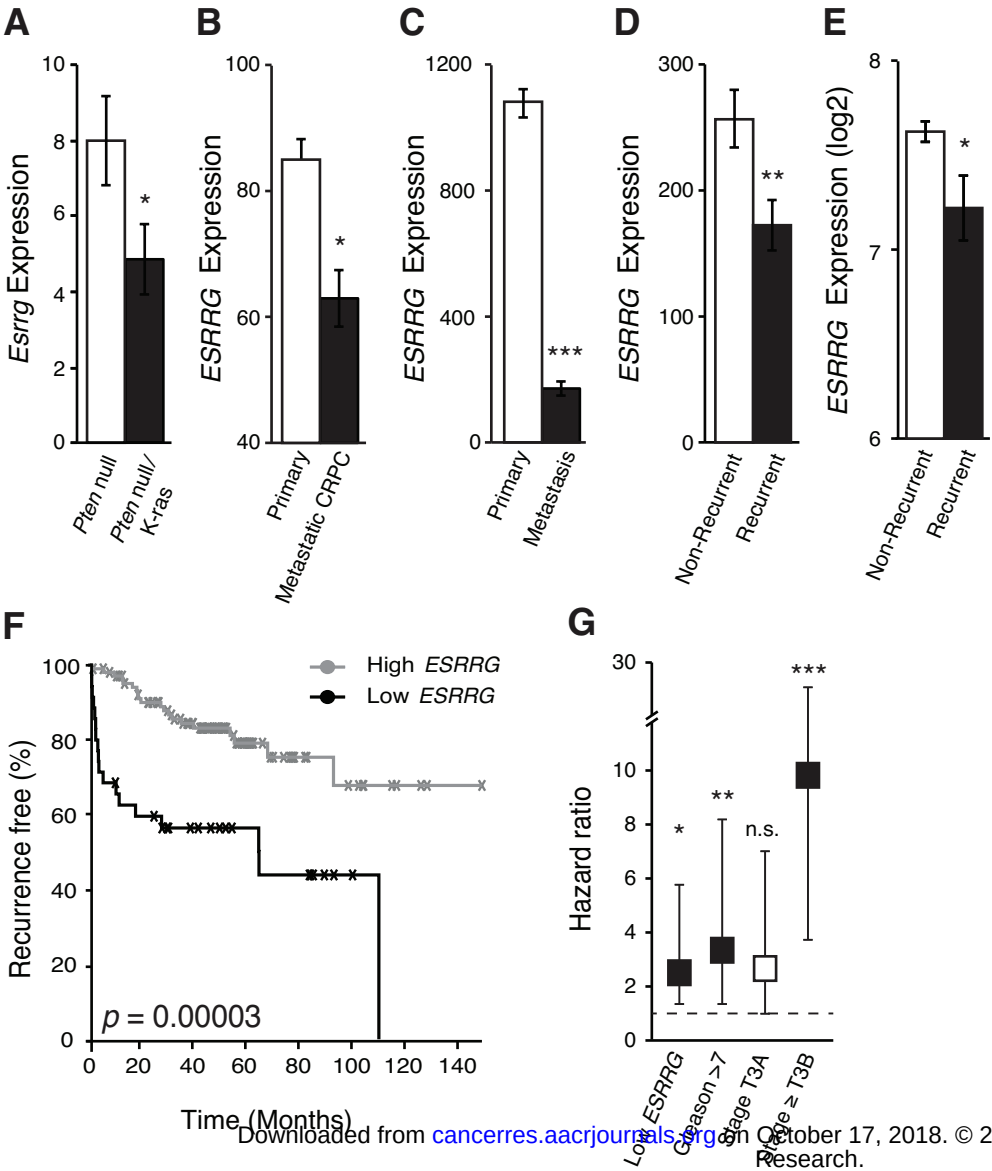

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Androgen-dependent repression of ERRγ reprograms metabolism in prostate cancer

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