Registered report: The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man

Nicole Perfito, Denise Chronscinski, Srujana Cherukuri, Fraser Tan, Elizabeth Iorns, Joelle Lomax

The Prostate Cancer Foundation-Movember Foundation Reproducibility Initiative (PCFMFRI) seeks to address growing concerns about reproducibility in scientific research by conducting replications of recent papers in the field of prostate cancer. This Registered Report describes the proposed replication plan of key experiments from “The Androgen Receptor Induces a Distinct Transcriptional Program in Castration-Resistant Prostate Cancer in Man” by Sharma and colleagues, published in *Cancer Cell* in 2013 (Sharma et al. 2013) . Of thousands of targets for the androgen receptor (AR), the authors elucidated a subset of 16 core genes that were consistently downregulated with castration and re-emerged with castration resistance. These 16 AR binding sites were distinct from those observed in cells in culture. The authors suggested that cellular context can have dramatic effects on downstream transcriptional regulation of AR binding sites. The present study will attempt to replicate Figure 7C by comparing gene expression of the 16 core genes identified by Sharma and colleagues in xenograft tumor tissue compared to androgen treated LNCaP cells in vitro. The Prostate Cancer Foundation-Movember Foundation Reproducibility Initiative is a collaboration betweenthe Prostate Cancer Foundation, the Movember Initiative, and Science Exchange, and the results of the replications will be published by *PeerJ*.

Registered report: The Androgen Receptor Induces a Distinct Transcriptional Program in Castration-Resistant Prostate Cancer in Man

Denise Chroscinski[1], Srujana Cherukuri[1], Prostate Cancer Foundation-Movember Foundation Reproducibility Initiative[†*]

[1] Noble Life Sciences, Inc. (Gaithersburg, MD, USA)
[†] The PCFMFRI core team consists of Elizabeth Iorns, Fraser Tan, Joelle Lomax and Nicole Perfito (Science Exchange, Palo Alto, California, USA).

* Correspondence to Nicole Perfito, nicole@scienceecxchange.com.

Abstract

The Prostate Cancer Foundation-Movember Foundation Reproducibility Initiative (PCFMFRI) seeks to address growing concerns about reproducibility in scientific research by conducting replications of recent papers in the field of prostate cancer. This Registered Report describes the proposed replication plan of key experiments from “The Androgen Receptor Inducesa Distinct Transcriptional Programin Castration-Resistant Prostate Cancer in Man” by Sharma and colleagues, published in *Cancer Cell* in 2013 (Sharma et al. 2013). Of thousands of targets for the androgen receptor (AR), the authors elucidated a subset of 16 core genes that were consistently downregulated with castration and re-emerged with castration resistance in both human and mouse prostate tumors. These 16 AR binding sites were distinct from those observed in cultured prostate tumor cells. The authors suggested that cellular context has dramatic effects on downstream transcriptional regulation of AR binding sites. The present study will attempt to replicate Figure 7C by comparing gene expression of the 16 core genes identified by Sharma and colleagues in xenograft tumor tissue compared to androgen-treated LNCaP cells *in vitro*. The Prostate Cancer Foundation-Movember Foundation Reproducibility Initiative is a collaboration between the Prostate Cancer Foundation, the Movember Initiative, and Science Exchange, and the results of the replications will be published by *PeerJ*.

Introduction

In tissues where it is expressed, the ligand-activated androgen receptor (AR) binds to DNA response elements and directs expression of cell- and tissue- specific genes (Kang et al. 2004, Wang et al. 2005). In order to form a functional transcription complex, AR employs co-regulator proteins and transcription factors to direct binding and influence transcription (Heinlein and Chang 2002). Prostate cancer (PC) cells depend on steroid hormones and AR to mediate oncogenic growth (Wang et al. 2009), and therefore, one of the key treatments for prostate cancer following surgery and radiation therapy is androgen deprivation therapy (ADT). ADT greatly reduces levels of the endogenous ligand for the androgen receptor and thus affects its target genes, hindering their ability to drive oncogenic growth (reviewed in Lian et al. 2015).

While initially effective, the disease often returns despite ADT; it is then termed castration-resistant prostate cancer (CRPC), an aggressive and fatal form of prostate cancer. The mechanisms of CRPC are not completely understood, but activation of the AR may play an important role in the transition to CRPC (Lin et al. 2013). Whole genome analyses point to genomic rearrangements and alterations in the transcriptional program particularly related to AR signaling in CRPC cells (Shtivelman et al. 2014). Functional outcomes of these alterations include increased AR mRNA and protein in CRPC cells (Yuan et al. 2014), the ability for other hormones to bind AR (Taplin et al. 1999), generation of ligand independent constitutively active AR variants (Ware et al. 2014, Ferraldeschi et al. 2015), and increased sensitivity of AR by posttranslational modifications (Guo et al. 2006).

In their 2013 *Cancer Cell* paper, Sharma and colleagues used chromatin immunoprecipitation sequencing (ChIP-seq) to identify genome-wide binding targets of AR in prostate tissue from patients with CRPC, ADT-responsive PC, or untreated PC, compared with benign prostate hyperplasia (Sharma et al. 2013). This analysis identified thousands of AR target genes. Of the 150 genes that were upregulated in untreated PC compared to CRPC, and downregulated with castration in PC xenografts, 16 core genes consistently re-emerged with castration resistance: *PECI*; *TNFSF10*; *ABHD12*; *XRCC3*; *MAD1L1*; *SEC61A1*; *GFM1*; *TSPAN13*; *STIL*; *TRMT12*; *EIF2B5*; *TM4SF1; NDUFB11; SLC26A2; AGR2; NT5DC3*. CRPC is often studied *in vitro* using cell lines derived from metastatic cancer cells (Wang et al. 2009, Yu et al. 2010, Massie et al. 2011), and previous authors have suggested PC cells in culture have different gene expression signatures than primary tumors (Yu et al. 2009, Long et al. 2014). Sharma and colleagues showed that genome-wide binding targets of the AR in tumor tissue differed from those in cultured cells. Expression patterns of the 16 core AR target genes were distinct from those observed in cells in culture, suggesting cellular context can have dramatic effects on downstream transcriptional regulation of AR binding sites.

In Figure 7C, expression levels of the 16 core genes were analyzed in xenograft tumor tissues that were determined to be either castration-sensitive or castration-resistance. Gene expression signatures in these tumors were compared to tumors from intact mice. The core genes were downregulated in castration-sensitive tumors compared to tumors from intact animals, and upregulated in castration-resistant tumors (Sharma et al. 2013). This is a key finding and will be replicated in Protocols 2 and 3. Another key finding of this study was that the expression patterns of the core genes in tumor tissues were distinct from their expression pattern in LNCaP cell culture. In order to replicate this key finding, we will also compare expression patterns of the core gene set in both castration-sensitive and castration-resistant tumors to the genetic signature of these genes in cultured LNCaP cells with or without androgen exposure *in vitro* in Protocols 1 and 3.

While no direct replications exist in the literature for genome-wide profiling of AR binding sites, parts of Sharma and colleagues' work have been reproduced in other studies. Tao and colleagues profiled AR target genes in cultured LNCaP cells but reported gene ontology only (Tao et al. 2014). Some of the 16 core AR-binding targets identified in Sharma, et al, have been

studied as markers for prostate cancer, either by mutation (*XRCC3*; Xuan et al. 2015) or increased splice variant expression (*AGR2*; Neeb et al. 2014). Some of the core 16 targets have been identified as targets for AR in breast cancer (*MAD1L1*; Mehta et al. 2015), in association with radiation-resistant lung adenocarcinoma cells (*TM4SF5*; Choi et al. 2014) and pancreatic ductal adenocarcinoma (*AGR2*; Mizuuchi et al. 2015). Lastly, an antibody targeting TM4SF1 reduced human prostate cancer cells in matrigel implants *in vivo* (Lin et al. 2014).

Materials & Methods

Unless otherwise noted, all protocol information was derived from the original paper, references from the original paper, or information obtained directly from the authors. An asterisk (*) indicates data or information provided by the PCFMFRI core team. A hashtag (#) indicates information provided by the replicating lab. All references to Figures are in reference to the original study.

Protocol 1: Stimulation of cultured LNCaP cells with androgen treatment

This protocol describes how to stimulate cultured LNCaP human prostate cancer cells with androgen in order to assess the expression level of the core set of 16 genes identified by Sharma and colleagues (Sharma et al. 2013). This is an additional experiment added by the PCFMFRI core team designed to probe for differences in gene signature between cultured cells and tumors *in vivo*, and is based upon work performed by Massie and colleagues (Massie et al. 2011). Cells produced in this protocol will be used for gene expression analysis in Protocol 3.

Sampling:

- Each experiment consists of two cohorts:
  - Cohort 1: untreated LNCaP cells
  - Cohort 2: LNCaP cells treated with R1881 (synthetic androgen)

- The experiment will be performed 3 times
  - This experiment is exploratory in nature, and thus no power calculations are necessary.

Materials and Reagents:

Reagent	Type	Manufacturer	Catalog #	Comments
LNCaP cells, clone FGC	Cells	ATCC	CRL-1740	Original unspecified
RPMI-1640	Media	ATCC	30-2001	
Fetal bovine serum (charcoal dextran-stripped)	Reagent	Sigma-Aldrich	F4135	Original source unspecified
6 cm tissue culture dishes	Labware	Will be left to the discretion of the replicating lab		Original source unspecified
R1881	Synthetic androgen	Sigma-Aldrich	R0908	
DMSO	Reagent	Sigma-Aldrich	D8418	Original source unspecified
TRIzol® Reagent	Reagent	Life Technologies	15596	Original not specified

Procedure:

Notes:

- All cells will be sent for mycoplasma testing and STR profiling

1. Plate 1 x 10^5 LNCaP cells in 6 cm tissue culture dishes and let grow overnight to adhere.

a. LNCaP cells are cultured in RPMI-1640 supplemented with 10% FBS at 37ºC/5% CO_2.
b. Prepare 2 separate plates; one for treatment, one for control.

2. Treat cells with R1881 for 3 days.
   a. For treatment, replace media with fresh media containing 1 nM R1881.
      i. R1881 is dissolved in DMSO at >10 mg/ml.
   b. For control, add vehicle (DMSO) only, at the same total volume as for treated cells.

3. #Lyse cells with TRIzol reagent, according to manufacturer's instructions.
   a. Use mechanical disruption (i.e., cell scraping) to dislodge cells, if necessary.

4. Immediately flash-freeze and store cell/TRIzol mixture at -80ºC until ready to perform RNA extraction in Protocol 3.

5. Repeat experiment an additional two independent times.

Deliverables:

- Data to be collected:
  - None applicable
- Samples delivered for further analysis:
  - Homogenized cells for use in Protocol 3, stored at -80ºC

Confirmatory analysis plan:

- See Protocol 3 for analysis of gene expression

Known differences from the original study:

- This experiment was not performed in the original study; it is an additional experiment added by the PCFMFRI core team in order to explore a the key finding that cultured cells possess a different AR-mediated gene signature for the core 16-gene set than *in situ* tumor tissue (Sharma et al. 2013).

Provisions for quality control:

All data obtained from the experiment - raw data, data analysis, control data and quality control data - will be made publicly available, either in the published manuscript or as an open access dataset available on the Open Science Framework (https://osf.io/84tu2/).

Protocol 2: Generation of castration-sensitive and castration-resistant LNCaP tumor xenografts

This protocol describes the procedure for creating LNCaP prostate cancer xenografts in rodents, and then uses surgical castration to differentiate tumor groups. Upon castration, tumors will either regress (castration-sensitive), or else continue to grow (castration-resistant). Tumor tissue from both intact and castrated mice (including tumors that are both castration-sensitive and -resistant) will be harvested at the end of the experiment for total RNA isolation and subsequent down-stream gene expression analysis in Protocol 3.

Sampling:

- The experiment will use at least 5 mice per group, for a power of at least 85.92%.
  - See Power Calculations for details.
  - To buffer against unexpected mouse deaths, 6 mice per cohort will be used.

- The experiment consists of two cohorts:
  - Cohort 1: non-castrated tumor-bearing mice
  - Cohort 2: castrated tumor-bearing mice
    - 70% of castrated mice develop castration-resistant tumors. Thus, to generate at least 6 castration-sensitive tumors (or 30% of total castrated mice) and at least 6 castration-resistant tumors, 22 mice will be castrated.
  - Total mice:
    - 28 mice injected with LNCaP cells
    - 22 injected mice will be surgically castrated

Materials and Reagents:

Reagent	Type	Manufacturer	Catalog #	Comments
HC-matrigel	Reagent	Corning	354262	Original catalogue number from BD unspecified
LNCaP cells, clone FGC	Cells	ATCC	CRL-1740	Original unspecified
NOD/SCID-gamma (NSG) mice	Mice	Jackson Labs		Original unspecified
1 mL insulin syringe with attached needle; 29G x 1/2 in.	Labware	BD Biosciences	329411	Original brand not specified
Trypsin	Reagent	Sigma-Aldrich	T6567	Original unspecified
PBS	Reagent	Life Technologies	14190	Original unspecified
FBS	Reagent	Sigma-Aldrich	R0908	Original unspecified
RPMI-1640	Synthetic androgen	ATCC	30-2001	
Ketamine	Drug	Will be left to the discretion of the replicating lab		Original unspecified
Xylazine	Drug	Will be left to the discretion of the replicating lab		Original unspecified
Isoflurane	Drug	Will be left to the discretion		Original unspecified

		of the replicating lab		
Isopropanol	Reagent	Sigma-Aldrich	W292907	Original unspecified
TRIzol® Reagent	Reagent	Life Technologies	15596	Original not specified
Silicon-carbide beads	Equipment	BioSpec	11079110 sc	Original unspecified
Retsch Mixer Mill	Equipment	Retsch	MM400	Original unspecified

Procedure:

Notes:

- All cells will be sent for mycoplasma testing and STR profiling

1. Culture LNCaP cells in RPMI-40 supplemented with 10% FBS at 37ºC/5% CO_2.
    a. Trypsinize to dissociate cells
    b. Centrifuge at ≤1,000 rpm and remove the supernatant
    c. Use a P200 pipette to gently dissociate the cells. Pipette up and down several times.
    d. Count cells
    e. Resuspend 2 x 10^6 dissociated cells in 0.1 ml cold PBS, then mix with an equal volume of cold Matrigel. Total volume should be 0.2 ml per injection.
        I. Approximately 6 x 10^7 cells will be needed for all 28 injections

2. Subcutaneously inject mice in the rear flank. Each mouse should receive a single injection.
    a. Mice should be microchipped prior to injection, so that they can be easily monitored throughout the duration of the study.
    b. Inject 0.2 ml cell/matrigel mixture per mouse subcutaneously into the flank of the mice using a 29G insulin syringe.

3. Measure tumor volume with manual calipers #twice weekly.
    a. Note time for tumors to form as well as tumor diameter and volume.
        i. Measurement 1 will be width, measurement 2 will be the length, and measurement 3 will be the height of the tumor.
        ii. Since the growth of the tumor is not uniform, we will use the formula $V = length \times width \times height \times 0.5326$ to obtain tumor volume.

4. Let tumors grow to approximately 100 mm^3. Tumor growth takes approximately 4 weeks.

5. Randomly assign mice to either the intact or castrate cohort (ratio ~1:5).
    a. #At the time of randomization (when the tumor growth in majority of mice is 100 mm^3), mice without any measurable tumors and those with tumor volume between approximately 400 - 500 mm^3 will be eliminated.
    b. #The remaining mice will be arranged in ascending order based on the tumor volume and group numbers will be assigned in a serpentine order in such way that the average tumor volume in each group will have equal or similar tumor volumes.

6. For the castration cohort, surgically castrate mice.
    a. #Castration procedure:

i. Anesthetize animal with 100 mg/kg ketamine / 5 mg/kg Xylazine
ii. Place on dorsal side facing the tail toward the surgeon. Prep the abdominal area by shaving the hair and swabbing with the surgical swab.
iii. An abdominal incision will be made and vas deferens and spermatic blood vessel is exteriorized and cauterized and testis tissue removed.
iv. The tissue is replaced into abdominal cavity and the incision will be closed with wound clips or absorbable sutures.
v. Castrated animals will be monitored daily for 7 days post castration.

b. Around 70% of castrated mice will develop castration resistant tumors, which, after an initial regression, will regrow.

7. Continue monitoring tumor volume in all cohorts #twice weekly until tumor reaches 10% of body weight.

8. Once tumor volume is ≥10% of body weight, sacrifice mouse and excise tumor.
   a. Euthanize mice under isoflurane anesthesia.
   b. Spray tumor-bearing area on the flank with 70% isopropanol.
   c. Make a small incision on the skin of the flank, and peel skin to expose the subcutaneous tumor.
   d. Excise tumor using surgical scissors and forceps.
   e. Excess non-tumor tissue will be cleaned from tumor and the tumor will be flash frozen in liquid nitrogen and stored in -80ºC until further processing.

9. Dissociate tissue #with bead homogenizer with 1.00mm silicon-carbide beads in TRIzol and store homogenized tissue at -80°C until RNA extraction in Protocol 3.

Deliverables:

- Data to be collected:
  - All mouse health records, including date of injection, date of castration, date of sacrifice, reason for sacrifice
  - All tumor volume measurements for all mice
- Samples delivered for further analysis:
  - #Homogenized tissue in TRIzol for use in Protocol 3, stored at -80ºC

Confirmatory analysis plan:

- See Protocol 3 for analysis of gene expression

Known differences from the original study:

All known differences in reagents and supplies are listed in the materials and reagents section above, with the originally used item listed in the comments section. Tumor volume will be measured twice weekly instead of daily as suggested by the replicating lab. All differences have the same capabilities as the original and are not predicted to alter experimental outcome.

Provisions for quality control:

All data obtained from the experiment - raw data, data analysis, control data and quality control data - will be made publicly available, either in the published manuscript or as an open access dataset available on the Open Science Framework (https://osf.io/84tu2/).

Protocol 3: Assessing expression of the core 16 gene-set by qRT-PCR

This protocol describes how to measure the expression levels of the core 16-gene set using semi-quantitative RT-PCR. This technique will be used to assess gene expression from samples generated in Protocols 1 and 2.

Sampling:

- This experiment consists of five cohorts (2 cohorts from Protocol 1 and 3 cohorts from Protocol 2):
  - Cohort 1: cDNA from untreated LNCaP cells [Protocol 1]
    - n = 3
  - Cohort 2: cDNA from LNCaP cells treated with R1881 [Protocol 1]
    - n = 3
  - Cohort 3: cDNA from tumors in intact mice [Protocol 2]
    - n = 6
  - Cohort 4: cDNA from castration-sensitive tumors in castrated mice [Protocol 2]
    - n = TBD
  - Cohort 5: cDNA from castration-resistant tumors in castrated mice [Protocol 2]
    - n = TBD

Materials and Reagents:

Reagent	Type	Manufacturer	Catalog #	Comments
RNeasy Kit	Reagent	Qiagen	74106	Original source unspecified
qScript cDNA synthesis kit	Reagent	Quanta Biosciences	95047	Original not specified
SYBR green mastermix	Reagent	Life Technologies	4472908	
Oligos for qRT-PCR	Primers	Synthesis left to the discretion of the replicating lab and will be recorded later		
StepOnePlus Real-Time PCR System	Equipment	Applied Biosystems		
384-well PCR Plates	Labware	Specific brand information will be left up to the discretion of the replicating lab and recorded later		

Procedure:

1. #Extract total RNA from TRIzol-preserved samples using RNeasy kit according to manufacturer's instructions.
   a. Report total concentration and purity of isolated total RNA.

2. Reverse transcribe #0.5 ug RNA from cell and tissue samples derived from Protocols 1 and 2 into cDNA using the #qScript cDNA synthesis kit according to the manufacturer's protocol.

3. Perform qPCR analysis of target genes in 384-well plates. *Run each reaction in triplicate.
    a. Use SYBR green mastermix with the following primer sets according to manufacturer's protocol:
        i. *PECI*:
            1. F: GCCGGTTGAACATGATCTTT
            2. R: ATGGGCTGAGGTTGTTTGTC
        ii. *TNFSF10*
            1. F: TGTGTCAGGGCTCTACTGTGA
            2. R: ATTCCCAGGGTAGGAGGAGA
        iii. *ABHD12*
            1. F: TAGCCCAGGCGTGTAATAGG
            2. R: CTGGCCTTGAAGCAACATCT
        iv. *XRCC3*
            1. F: GCAATCACAGCCAGAACAGA
            2. R: CAGAAGCAGAGTGTCCCACA
        v. *MAD1L1*
            1. F: GCACCCCTGTTGTTTTCATT
            2. R: ATGCCTGTTCCTTTGTGACC
        vi. *SEC61A1*
            1. Replicating lab will design and optimize primers for this gene
        vii. *GFM1*
            1. F: AAAAGGACTCCCTTCCCTCA
            2. R: ACGCGAGGAAAAAGAGAGTG
        viii. *TSPAN13*
            1. Replicating lab will design and optimize primers for this gene
        ix. *STIL*
            1. F: TCGACCAATCCCAAGTCTTC
            2. R: ATAGAGCACTTCCGGCTTCA
        x. *TRMT12*
            1. F: CTCAAGCAAGCGCATCAATA
            2. R: GGGCTTCCCACTTTCTCTCT
        xi. *EIF2B5*
            1. F: CAGACAGATCGGGTTCCAAT
            2. R: TTCCATTGAGCGCTGATTTT
        xii. *TM4SF1*
            1. F: TGCATTCATTTGGATTGGAA
            2. R: GAAAATCCGACAACCTGGAA
        xiii. *NDUFB11*
            1. Replicating lab will design and optimize primers for this gene
        xiv. *SLC26A2*
            1. F: GGAAAGGGAAGGAAAGGAAG
            2. R: TAGCCACAGCCAGTCACATC
        xv. *AGR2*
            1. F: CAGCCATTCAAATCCCTTGT
            2. R: AAGAGTTCGTGGGGAAATCA
        xvi. *NT5DC3*
            1. F: ATGCACATCTTGGGAAGGTC
            2. R: TCCCTCCCCTTTTCCTCTTA
        xvii. Actin control primers;
            1. Replicating lab will design and optimize primers for this gene

b. Reaction mixture:
    i. Set up according to the manufacturer's protocols for SYBR Green mastermix
c. Run qPCR reactions on an #ABI StepOnePlus qPCR machine with the following cycling parameters:
    i. 2 min at 50°C
    ii. 10 min at 95°C
    iii. 40 cycles of:
        1. 15 sec at 95°C
        2. 1 min at 60°C
d. Analyze qPCR data using the ΔCt method normalized using the Actin control.

Deliverables:

- Data to be collected:
    - Quality control data for total RNA and synthesized cDNA
        - A_{280}/A_{260} and A_{260}/A_{230} ratios for both
    - Efficiency calculations for each primer pair
    - Raw and normalized (to control gene) qRT-PCR values
    - Data analyzed with the ΔΔCt method

Confirmatory analysis plan:

This replication attempt will perform the statistical analyses listed below, compute the effects sizes, compare them against the reported effect size in the original paper and use a meta-analytic approach to combine the original and replication effects, which will be presented as a Forest plot.

- Statistical Analysis of the Replication Data:
    - Comparison of average gene expression for the core 16 genes in xenografts from full, castration-sensitive and castration-resistant tumors
        - One-way ANOVA followed by planned pairwise comparisons using the Bonferroni correction to account for multiple comparisons:
            - Full vs. castration-sensitive
            - Castration-sensitive vs. castration-resistant

- Additional Statistical Analysis of the Replication Data:
    - Comparison of gene expression averaged within each sample (in xenografts and in cultured cells) for the core 16 genes.
        - Two-way ANOVA with Cell Type (Xenograft vs Culture) and Treatment (Androgen exposure/Full or Control/Castration-sensitive as main effects followed by planned pairwise comparisons using the Bonferroni correction to account for multiple comparisons:
            - Control LNCaP cells vs. R1881 treated LNCaP cells
            - Full xenograft vs. R1881 treated LNCaP cells
            - Castration-sensitive xenograft vs. Control LNCaP cells

Known differences from the original study:

The original study used multiple methods for gene expression analysis, including Illumina expression arrays and qRT-PCR. This replication will only use qRT-PCR to analyze and compare gene expression of the core 16-gene set. All known differences in reagents and supplies are listed in the materials and reagents section above, with the originally used item

listed in the comments section. All differences have the same capabilities as the original and are not predicted to alter experimental outcome.

Provisions for quality control:

All data obtained from the experiment - raw data, data analysis, control data and quality control data - will be made publicly available, either in the published manuscript or as an open access dataset available on the Open Science Framework (https://osf.io/84tu2/).

Power Calculations

Protocol 1:

No power calculation necessary.

Protocol 2:

Summary of original data

- Note: data were estimated from published heat map.
  - A greyscale intensity value was generated for each gene in each sample using Image J. That value was normalized with respect to white (set to 0) and color (positive numbers for red, negative numbers for blue).
  - These scores were averaged across all 16 genes to generate a total score for each sample.
  - The samples in each group were averaged and a mean and SD generated.
  - See the Data Estimation worksheet for details.

Figure 7C	Mean score	SD	N
Full	0.63	0.10	4
Castration-sensitive	-0.79	0.56	4
Castration-resistant	0.32	0.44	4

Test family

- One-way ANOVA on the mean scores per group followed by Bonferroni corrected comparisons on the following tumor groups:
  - Full vs. castration-sensitive
  - Castration-sensitive vs. castration-resistant

Power Calculations

- Power calculations were performed using GraphPad PRISM v6 for Mac and G*Power v. 3.1.7 (Faul et al. 2007).

One-way ANOVA						
F(2,10)	Partial eta^2	α	Effect size *f*	Power	Total samples	N per group
12.86	0.740783	0.05	1.690495	93.81%	9	3*

* With 5 samples per group, based on planned comparisons, achieved power is 99.96%.

Bonferroni corrected *t*-tests

Group 1	Group 2	Effect size d	α	Power	N per group
Full	Castration-sensitive	3.51191	0.025	88.80%	3*
Castration-sensitive	Castration-resistant	2.19747	0.025	85.92%	5

*With 5 samples per group, achieved power is 99.78%.

Protocol 3:

No power calculation necessary.

Acknowledgements:

The PMFRI core team would like to thank the original authors, in particular Dr. Charlie Massie and Dr. David Neal, for generously sharing critical information as well as reagents to ensure the fidelity and quality of this replication attempt.

Competing Interests:

We disclose that EI, FT, NP and JL are employed by and hold shares in Science Exchange Inc. The experiments presented in this manuscript will be conducted by Noble Life Sciences, Inc., which is a Science Exchange lab. No other authors disclose conflicts of interest related to this manuscript.

Funding:

The PCF Movember Foundation Reproducibility Initiative is funded by the Prostate Cancer Foundation and the Movember Foundation. The funders had no role in study design or the decision to submit the work for publication.

References

Choi, SI, Kim, SY, Lee, J, Cho, EW and Kim, IG (2014). TM4SF4 overexpression in radiation-resistant lung carcinoma cells activates IGF1R via elevation of IGF1. *Oncotarget* **5**(20): 9823-9837.

Faul, F, Erdfelder, E, Lang, AG and Buchner, A (2007). G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* **39**(2): 175-191.

Ferraldeschi, R, Welti, J, Luo, J, Attard, G and de Bono, JS (2015). Targeting the androgen receptor pathway in castration-resistant prostate cancer: progresses and prospects. *Oncogene* **34**(14): 1745-1757. 10.1038/onc.2014.115

Guo, Z, Dai, B, Jiang, T, Xu, K, Xie, Y, Kim, O, Nesheiwat, I, Kong, X, Melamed, J, Handratta, VD, Njar, VC, Brodie, AM, Yu, LR, Veenstra, TD, Chen, H and Qiu, Y (2006). Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell* **10**(4): 309-319. 10.1016/j.ccr.2006.08.021

Heinlein, CA and Chang, C (2002). Androgen receptor (AR) coregulators: an overview. *Endocr Rev* **23**(2): 175-200. 10.1210/edrv.23.2.0460

Kang, Z, Janne, OA and Palvimo, JJ (2004). Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. *Mol Endocrinol* **18**(11): 2633-2648. 10.1210/me.2004-0245

Lian, F, Sharma, NV, Moran, JD and Moreno, CS (2015). The biology of castration-resistant prostate cancer. *Curr Probl Cancer* **39**(1): 17-28. 10.1016/j.currproblcancer.2014.11.004

Lin, CI, Merley, A, Sciuto, TE, Li, D, Dvorak, AM, Melero-Martin, JM, Dvorak, HF and Jaminet, SC (2014). TM4SF1: a new vascular therapeutic target in cancer. *Angiogenesis* **17**(4): 897-907. 10.1007/s10456-014-9437-2

Lin, D, Gout, PW and Wang, Y (2013). Lessons from in-vivo models of castration-resistant prostate cancer. *Curr Opin Urol* **23**(3): 214-219. 10.1097/MOU.0b013e32835e9f07

Long, Q, Xu, J, Osunkoya, AO, Sannigrahi, S, Johnson, BA, Zhou, W, Gillespie, T, Park, JY, Nam, RK, Sugar, L, Stanimirovic, A, Seth, AK, Petros, JA and Moreno, CS (2014). Global transcriptome analysis of formalin-fixed prostate cancer specimens identifies biomarkers of disease recurrence. *Cancer Res* **74**(12): 3228-3237. 10.1158/0008-5472.CAN-13-2699

Massie, CE, Lynch, A, Ramos-Montoya, A, Boren, J, Stark, R, Fazli, L, Warren, A, Scott, H, Madhu, B, Sharma, N, Bon, H, Zecchini, V, Smith, DM, Denicola, GM, Mathews, N, Osborne, M, Hadfield, J, Macarthur, S, Adryan, B, Lyons, SK, Brindle, KM, Griffiths, J, Gleave, ME, Rennie, PS, Neal, DE and Mills, IG (2011). The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. *EMBO J* **30**(13): 2719-2733. 10.1038/emboj.2011.158

514 Mehta, J, Asthana, S, Mandal, CC and Saxena, S (2015). A molecular analysis provides novel
515 insights into androgen receptor signalling in breast cancer. *PLoS One* **10**(3): e0120622.
516 10.1371/journal.pone.0120622
517
518 Mizuuchi, Y, Aishima, S, Ohuchida, K, Shindo, K, Fujino, M, Hattori, M, Miyazaki, T, Mizumoto,
519 K, Tanaka, M and Oda, Y (2015). Anterior gradient 2 downregulation in a subset of
520 pancreatic ductal adenocarcinoma is a prognostic factor indicative of epithelial-
521 mesenchymal transition. *Lab Invest* **95**(2): 193-206. 10.1038/labinvest.2014.138
522
523 Neeb, A, Hefele, S, Bormann, S, Parson, W, Adams, F, Wolf, P, Miernik, A, Schoenthaler, M,
524 Kroenig, M, Wilhelm, K, Schultze-Seemann, W, Nestel, S, Schaefer, G, Bu, H, Klocker,
525 H, Nazarenko, I and Cato, AC (2014). Splice variant transcripts of the anterior gradient 2
526 gene as a marker of prostate cancer. *Oncotarget* **5**(18): 8681-8689.
527
528 Sharma, NL, Massie, CE, Ramos-Montoya, A, Zecchini, V, Scott, HE, Lamb, AD, MacArthur, S,
529 Stark, R, Warren, AY, Mills, IG and Neal, DE (2013). The androgen receptor induces a
530 distinct transcriptional program in castration-resistant prostate cancer in man. *Cancer*
531 *Cell* **23**(1): 35-47. 10.1016/j.ccr.2012.11.010
532
533 Shtivelman, E, Beer, TM and Evans, CP (2014). Molecular pathways and targets in prostate
534 cancer. *Oncotarget* **5**(17): 7217-7259.
535
536 Tao, S, He, H and Chen, Q (2014). ChIP-seq analysis of androgen receptor in LNCaP cell line.
537 *Mol Biol Rep* **41**(9): 6291-6296. 10.1007/s11033-014-3511-0
538
539 Taplin, ME, Bubley, GJ, Ko, YJ, Small, EJ, Upton, M, Rajeshkumar, B and Balk, SP (1999).
540 Selection for androgen receptor mutations in prostate cancers treated with androgen
541 antagonist. *Cancer Res* **59**(11): 2511-2515.
542
543 Wang, Q, Carroll, JS and Brown, M (2005). Spatial and temporal recruitment of androgen
544 receptor and its coactivators involves chromosomal looping and polymerase tracking.
545 *Mol Cell* **19**(5): 631-642. 10.1016/j.molcel.2005.07.018
546
547 Wang, Q, Li, W, Zhang, Y, Yuan, X, Xu, K, Yu, J, Chen, Z, Beroukhim, R, Wang, H, Lupien, M,
548 Wu, T, Regan, MM, Meyer, CA, Carroll, JS, Manrai, AK, Janne, OA, Balk, SP, Mehra, R,
549 Han, B, Chinnaiyan, AM, Rubin, MA, True, L, Fiorentino, M, Fiore, C, Loda, M, Kantoff,
550 PW, Liu, XS and Brown, M (2009). Androgen receptor regulates a distinct transcription
551 program in androgen-independent prostate cancer. *Cell* **138**(2): 245-256.
552 10.1016/j.cell.2009.04.056
553
554 Ware, KE, Garcia-Blanco, MA, Armstrong, AJ and Dehm, SM (2014). Biologic and clinical
555 significance of androgen receptor variants in castration resistant prostate cancer. *Endocr*
556 *Relat Cancer* **21**(4): T87-T103. 10.1530/ERC-13-0470
557
558 Xuan, G, Hui, Y and Fang, H (2015). The association of XRCC3 Thr241Met genetic variant with
559 risk of prostate cancer: a meta-analysis. *Afr Health Sci* **15**(1): 117-122.
560 10.4314/ahs.v15i1.16
561
562 Yu, J, Yu, J, Mani, RS, Cao, Q, Brenner, CJ, Cao, X, Wang, X, Wu, L, Li, J, Hu, M, Gong, Y,
563 Cheng, H, Laxman, B, Vellaichamy, A, Shankar, S, Li, Y, Dhanasekaran, SM, Morey, R,
564 Barrette, T, Lonigro, RJ, Tomlins, SA, Varambally, S, Qin, ZS and Chinnaiyan, AM

(2010). An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. *Cancer Cell* **17**(5): 443-454. 10.1016/j.ccr.2010.03.018

Yu, SQ, Lai, KP, Xia, SJ, Chang, HC, Chang, C and Yeh, S (2009). The diverse and contrasting effects of using human prostate cancer cell lines to study androgen receptor roles in prostate cancer. *Asian J Androl* **11**(1): 39-48. 10.1038/aja.2008.44

Yuan, X, Cai, C, Chen, S, Chen, S, Yu, Z and Balk, SP (2014). Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. *Oncogene* **33**(22): 2815-2825. 10.1038/onc.2013.235