Androgen-dependent alternative mRNA isoform expression in prostate cancer cells [version 1; referees: 2 approved]

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Abstract

Background: Androgen steroid hormones are key drivers of prostate cancer. Previous work has shown that androgens can drive the expression of alternative mRNA isoforms as well as transcriptional changes in prostate cancer cells. Yet to what extent androgens control alternative mRNA isoforms and how these are expressed and differentially regulated in prostate tumours is unknown.

Methods: Here we have used RNA-Seq data to globally identify alternative mRNA isoform expression under androgen control in prostate cancer cells, and profiled the expression of these mRNA isoforms in clinical tissue.

Results: Our data indicate androgens primarily switch mRNA isoforms through alternative promoter selection. We detected 73 androgen regulated alternative transcription events, including utilisation of 56 androgen-dependent alternative promoters, 13 androgen-regulated alternative splicing events, and selection of 4 androgen-regulated alternative 3′ mRNA ends. 64 of these events are novel to this study, and 26 involve previously unannotated isoforms. We validated androgen dependent regulation of 17 alternative isoforms by quantitative PCR in an independent sample set. Some of the identified mRNA isoforms are in genes already implicated in prostate cancer (including LIG4, FDFT1 and RELAXIN), or in genes important in other cancers (e.g. NUP93 and MAT2A). Importantly, analysis of transcriptome data from 497 tumour samples in the TGCA prostate adenocarcinoma (PRAD) cohort identified 13 mRNA isoforms (including TPD52, TACC2 and NDUFS3) that are differentially regulated in localised prostate cancer relative to normal tissue, and 3 (OSBPL1A, CLK3 and TSC22D3) which change significantly with Gleason grade and tumour stage.

Conclusions: Our findings dramatically increase the number of known androgen regulated isoforms in prostate cancer, and indicate a highly complex...
androgen regulated isoforms in prostate cancer, and indicate a highly complex response to androgens in prostate cancer cells that could be clinically important.

**Keywords**
Androgens, AR, prostate cancer, alternative splicing, alternative promoters, alternative 3' ends, transcription, mRNA isoforms
Introduction

A single human gene can potentially yield a diverse array of alternative mRNA isoforms, thereby expanding both the repertoire of gene products and subsequently the number of alternative proteins produced. mRNAs with different exon combinations are transcribed from most (up to 90%) human genes, and can generate variants that differ in regulatory untranslated regions, or encode proteins with different sub-cellular localisations and functions.\textsuperscript{1,3} Altered splicing patterns have been suggested as a new hallmark of cancer cells\textsuperscript{4-8}, and in prostate cancer there is emerging evidence that expression of specific mRNA isoforms derived from cancer-relevant genes may contribute to disease progression\textsuperscript{9-11}.

Androgen steroid hormones and the androgen receptor (AR) play a key role in the development and progression of prostate cancer, with alternative splicing enabling cancer cells to produce constitutively active ARs\textsuperscript{11-13}. The AR belongs to the nuclear receptor superfamily of transcription factors, and is essential for prostate cancer cell survival, proliferation and invasion\textsuperscript{14-16}. Classically, androgen binding promotes AR dimerization and its translocation to the nucleus, where it acts as either a transcriptional activator or a transcriptional repressor to dictate prostate specific gene expression patterns\textsuperscript{17-23}. The major focus for prostate cancer therapeutics has been to reduce androgen levels through androgen deprivation therapy (ADT), either with inhibitors of androgen synthesis (for example, abiraterone) or with antagonists that prevent androgen binding to the AR (such as bicalutamide or enzalutamide)\textsuperscript{24}. Although ADT is usually initially effective, most patients ultimately develop lethal castrate resistant disease for which there are limited treatment options\textsuperscript{11,12}.

Androgens and other steroid hormones have also been associated with alternative splicing. Recent RNA-sequencing-based analysis of the androgen response of prostate cancer cells grown in vitro and within patients following ADT identified a set of 700 genes whose transcription is regulated by the AR in prostate cancer cells\textsuperscript{25}. However, in addition to regulating transcriptional levels, steroid hormone receptors can control exon content of mRNA\textsuperscript{26-29}. In prostate cancer androgens can modulate the expression of mRNA isoforms via pre-mRNA processing and promoter selection\textsuperscript{30}. The AR can recruit the RNA binding proteins Sam68 and p68 as cofactors to influence alternative splicing of specific genes, and studies using minigenes driven from steroid responsive promoters indicate that the AR can affect both the transcriptional activity and alternative splicing of a subset of target genes\textsuperscript{31,32}. Other steroid hormones also coordinate both transcription and splicing decisions\textsuperscript{33}. The thyroid hormone receptor (TR) is known to play a role in coordinating the regulation of transcription and alternative splicing\textsuperscript{34}, and the oestrogen receptor (ER) can both regulate alternative promoter selection and induce alternative splicing of specific gene sets that can influence breast cancer cell behaviour\textsuperscript{11,35-37}.

In previous work we used exon level microarray analysis to identify 7 androgen dependent changes in mRNA isoform expression\textsuperscript{38}. However, to what extent androgen-regulated mRNA isoforms are expressed in clinical prostate cancer is unclear. To address this, here we have used RNA-Sequencing data to globally profile alternative isoform expression in prostate cancer cells exposed to androgens, and correlated the results with transcriptomic data from clinical tissue. Our findings increase the number of known AR regulated mRNA isoforms by 10 fold and imply that pre-mRNA processing is an important mechanism through which androgens regulate gene expression in prostate cancer.

Methods

Cell culture

Cell culture was as described previously\textsuperscript{25,39}. All cells were grown at 37°C in 5% CO\textsubscript{2}. LNCaP cells (CRL-1740, ATCC) were maintained in RPMI-1640 with L-Glutamine (PAW Laboratories, R15-802) supplemented with 10% Fetal Bovine Serum (FBS) (PAW Laboratories, A15-101). For androgen treatment of cells, medium was supplemented with 10% dextran charcoal stripped FBS (PAW Laboratories, A15-119) to produce a steroid-deplete medium. Following culture for 72 hours, 10 nM synthetic androgen analogue methyltrienolone (R1881) (Perkin-Elmer, NLP00505SMG) was either added (Androgen +) or absent (Steroid deplete) for the times indicated.

RNA-seq analysis

RNA-seq transcript expression analysis of previously generated data\textsuperscript{25} was performed according to the Tuxedo protocol\textsuperscript{40}. All reads were first mapped to human transcriptome/genome (build hg19) with TopHat\textsuperscript{41}/Bowtie\textsuperscript{42}, followed by per-sample transcript assembly with Cufflinks\textsuperscript{43}. The mapped data was processed with Cuffmerge, Cuffdiff and Cuffcompare, followed by extraction of significantly differentially expressed genes/isoforms; expression changes between cells grown with androgen and cells grown without androgens were assessed. Reference files for the human genome (UCSC build hg19) were downloaded from the Cufflinks pages: (UCSC-hg19 package from June 2012 was used.). The software versions used for the analysis were: TopHat v1.4.1, SAM tools Version: 0.1.18 (r982:295), bowtie version 0.12.8 (64-bit) and cufflinks v1.3.0 (linked against Boost version 104000). The Tuxedo protocol\textsuperscript{40} was carried out as follows: For steps 1–5, no parameters (except for paths to input/output files) were altered. In step 15, additional switches -s, -R, and -C were used when running cuffcompare. Steps 16–18 (extraction of significant results) were performed on the command line.

RNA extraction, RT–PCR and real-time PCR

Cells were harvested and total RNA extracted using TRIzol (Invitrogen, 15596-026) according to manufacturer’s instructions. RNA was treated with DNase 1 (Ambion, AM2222) and cDNA was generated by reverse transcription of 500ng of total RNA using the Superscript VILO cDNA synthesis kit (Invitrogen, 11754-050). Alternative events were analysed by either reverse transcriptase PCR or real-time PCR. Exon profiles were monitored and quantified using the Qiagen capillary electrophoresis system (Qiagen) and percentage inclusion was calculated as described previously\textsuperscript{46}. Real time PCR was performed in triplicate on cDNA using SYBR® Green PCR Master Mix
Global identification of androgen-dependent mRNA isoform production in prostate cancer cells predicts a major role for alternative promoter utilisation

We analysed previously published RNA-Seq data from LNCaP cells\(^9\) to globally profile how frequently androgens drive production of alternative mRNA isoforms in prostate cancer cells. This analysis identified a group of 73 androgen regulated alternative mRNA isoforms, which could be validated by visualisation on the UCSC Genome Browser\(^9\) (Table 1). 64 AR regulated mRNA isoforms were novel to this study. Experimental validation in an independent RNA sample set using RT-PCR confirmed 17/17 of these alternative events at the mRNA level (Supplementary Figure 1). 73% of genes (53/73) with identified alternative androgen regulated mRNA isoforms also changed their overall expression levels in response to androgens (Table 2). Some of the androgen regulated alternative events are in genes are already implicated in either prostate cancer or other cancer types (summarised in Table 3). However, Gene Ontology analysis of these 73 genes did not identify any significantly enriched biological processes.

The 73 identified mRNA isoforms were generated via androgen-regulated utilisation of 56 alternative promoters, 4 alternative 3’ ends and 13 alternative splicing events (Figure 1A).
Table 1. Details of the 73 androgen regulated mRNA isoforms identified in prostate cancer cells.

| Gene   | Event type | Position (hg19) | RefSeq       | Position (hg19) | RefSeq       | Change with androgens | PCR Validation | Predicted to change protein? | Isoform 1 ID   | Isoform 2 ID   | Comparable? |
|--------|------------|----------------|--------------|----------------|--------------|-----------------------|----------------|-------------------------------|----------------|----------------|-------------|
| LIG4   | Alternative | chr13:108859792-108807016 | NM_001098268.1 | chr13:108859792-108807130 | NM_002312.3 | Induction of promoter 2 | Yes (Qiagen) | No (5' UTR) | uc001vqr.2 | uc001vqr.2 | Yes         |
| TACC2  | Alternative | chr10:123748689-124014060 | NM_005862.3 | chr10:123748554-124014060 | NM_001291879.1 | Repression of promoter 1 | Yes (Qiagen) | Yes | uc001vqr.2 | uc001vqr.2 | Yes         |
| TPDS2  | Alternative | chr8:80947103-81083894 | NM_001287144.1 | chr8:80947103-810993066 | NM_001025252.2 | Induction of promoter 2 | Yes (Qiagen) | Yes | uc003ybr.1 | uc003ybr.1 | Yes         |
| NUP93  | Alternative | chr16:56764017-5687861 | NM_014669.4 | chr16:56815704-5687861 | NM_001242795.1 | Induction of promoter 1 | Yes (SYBR) | Yes | uc002ekb.2 | uc002ekb.2 | Yes         |
| RL1    | Alternative | chr5:5334932-5339873 | NM_006911.3 | chr5:5335270-5339396 | Not annotated | Repression of promoter 1 | Yes (Qiagen) | Yes (change from non-coding) | uc003zb.1 | Not annotated | No          |
| AP2S1  | Alternative | chr19:47341415-47354252 | NM_00501076.1 | chr19:47341415-47335347 | NM_001301076.1 | Induction of promoter 2 | Yes (SYBR) | Yes | uc002pul.1 | Not annotated | No          |
| RL2    | Alternative | chr9:5299866-5304611 | NM_005059.3 | chr9:5299860-5304222 | Not annotated | Repression of promoter 1 | Yes (Qiagen) | Yes (from non-coding) | uc003zi.1 | Not annotated | No          |
| PIK3R1 | Alternative | chr5:67511584-67597649 | NM_181523.2 | chr5:67584252-67597649 | NM_181524.1 | Repression of promoter 2 | Yes (SYBR) | Yes | uc003va.2 | uc003vjc.2 | Yes         |
| MAPRE2 | Alternative | chr18:32556892-32723432 | NM_001943826.2 | chr18:32561324-326723432 | NM_014266.8 | Switch to promoter 2 | Yes (Qiagen) | Yes | uc010xgb.1 | uc009vyl.2 | Yes         |
| NDUFAF4| Alternative | chr6:97337817-97345767 | NM_014165.3 | chr6:9733727-97345368 | Not annotated | Repression of promoter 2 | Yes (Qiagen) | Yes (change from non-coding) | uc003pov.2 | Not annotated | No          |
| DCXR   | Alternative | chr17:79993757-79995573 | NM_002686.3 | chr17:79993757-79995217 | Not annotated | Repression of promoter 2 | Yes (Qiagen) | Yes | uc002kdq.2 | Not annotated | No          |
| PEX10  | Alternative | chr1:2336241-2344010 | NM_006217.3 | Not annotated | Not annotated | Switch to promoter 2 | Yes (Qiagen) | Yes | uc001ajh.2 | Not annotated | No          |
| SNAPC2 | Alternative | chr19:7985194-7988136 | NM_003083.3 | chr19:7985687-7988136 | NR_003701.7 | Switch to promoter 2 | Yes (SYBR) | Yes (change from non-coding) | uc002miv.1 | uc002miv.1 | Yes         |
| ATP6V0D1| Alternative | chr16:674731917-67475898 | NM_004691.4 | chr16:67471931-67475338 | Not annotated | Repression of promoter 2 | Yes | uc002ete.1 | Not annotated | No          |
| ARRC1  | Alternative | chr9:140500092-140508912 | NM_001317968.1 | chr9:140506874-140509793 | Not annotated | Induction of promoter 2 | Yes (change from non-coding) | Yes (SYBR) | uc004cnp.1 | Not annotated | No          |
| DENND1A| Alternative | chr9:126141933-126692417 | NM_0020946.1 | chr9:126143408-126586780 | Not annotated | Repression of promoter 2 | Yes | uc004bnz.1 | Not annotated | No          |
| Gene    | Isoform 1 | Isoform 2 | Predicted to change protein | PCR Validation | Change with antigens | TCGA PRAD | Isoform 2 ID |
|---------|-----------|-----------|----------------------------|----------------|----------------------|-----------|--------------|
| KILH3   | chr16:84964217-84964226 | NM_024731.3 | Yes | annotated | uc002bff.1 | F1000Research 2018, 7:1189 Last updated: 18 SEP 2018 | uc003bjq.1 |
| RAB3I   | chr11:51646781-51646799 | NM_001271686.1 | Yes | annotated | NM_001073070-155555592 | Not annotated | uc000wiv.2 |
| ACER3   | chr17:21742011-21742021 | NM_0191682.1 | Yes | annotated | NM_001099659.2 | uc002ayg.3 | uc000ayg.1 |
| CEBPLA  | chr11:75351917-75352021 | NM_0020697.3 | Yes | annotated | NM_0010803.4 | uc002kve.2 | uc003gvu.3 |
| TRIM16  | chr19:19710685-19710694 | NM_0000670.3 | Yes | annotated | NM_002ayy.1 | uc000ayy.3 | uc000ayy.2 |
| VSIG3L  | chr19:55555592-55555593 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| SEPT5   | chr17:1970685-1970694 | NM_0000542.3 | Yes | annotated | NM_00014963.3 | uc002ayg.3 | uc000ayg.1 |
| HNNGCR  | chr7:7467306-7467306 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| RHDH3   | chr9:19555592-19555593 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| GFRIN2  | chr10:1496346-1496347 | NM_0000542.3 | Yes | annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| CIK3    | chr15:74900713-74900714 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| TRAPD6  | chr15:74900713-74900714 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| CDIP1   | chr18:3919080-3919081 | NM_00109789.2 | No | Not annotated | uc001ayg.1 | uc002ayg.3 | uc000ayg.1 |
| YIF1B   | chr18:3919080-3919081 | NM_00109789.2 | No | Not annotated | uc001ayg.1 | uc002ayg.3 | uc000ayg.1 |
| LINK2   | chr22:31069560-31069561 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| TSC22D3 | chr18:3919080-3919081 | NM_00109789.2 | No | Not annotated | uc001ayg.1 | uc002ayg.3 | uc000ayg.1 |
| ALDH1A3 | chr15:10149871-10149872 | NM_00109789.2 | No | Not annotated | uc001ayg.1 | uc002ayg.3 | uc000ayg.1 |
| TRABD2  | chr22:31069560-31069561 | NM_00288.5 | No | Not annotated | NM_001014957.1 | uc002ayg.3 | uc000ayg.1 |
| Gene   | Event type             | Position (hg19) | RefSeq    | Isoform 1 ID | Isoform 2 ID | TCGA PRAD | Isoform 1 D | Isoform 2 D | Comparable? | Predicted to change protein? | Change with antitgens | PCR Validation | Induction of promoter 1 | Induction of promoter 2 | RefSeq | Isoform 2 | Induction of promoter 1 | Induction of promoter 2 |
|--------|------------------------|-----------------|-----------|--------------|--------------|-----------|-------------|-------------|-------------|-----------------------------|----------------------|-----------------|--------------------------|--------------------------|---------|----------|--------------------------|--------------------------|
| GMFB   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| MLSTB  | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| TLE3   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| UBA1   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| TMGC1B | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| FDF1   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| GRPB1  | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| NCAPD3 | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| KLC2   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| RAPP1   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| TMEM79 | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| NHA1   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| ZNF32  | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| C10orf3| Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| UBEZ3  | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| KRT8   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| ELOVL1 | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| ROG1   | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| SQRF3B | Alternative promoter   | chr1:54943134-  | NM_001124.2| uc011cet.1   | uc002asn.2   | u0719849w1 | u000557p2   | u000557p2   | No           | No (5' UTR) | Switch to promoter 2        | No                    | Yes              | No                       | No                       | No      | No       | Not annotated            | annotated               |
| Gene       | Event type                      | Position (hg19) | RefSeq        | Change with androgens | PCR Validation | Predicted to change protein? | Isoform 1 ID | Isoform 2 ID | Comparable? |
|------------|---------------------------------|----------------|---------------|-----------------------|----------------|-------------------------------|--------------|--------------|-------------|
| MAT2A      | Alternative 3' end              | chr2:85766101-  | NM_005911.5   | Repression of isoform 2 | Yes (Qiavel)   | Yes                           | uc002spr.2   | uc010ysr.1   | Yes         |
|            |                                 | 8577240         |               |                       |                |                               |              |              |             |
| CNNM2      | Alternative 3' end              | chr10:104678075-| NM_199077.2   | Induction of isoform 1 | Yes (SYBR)     | Yes                           | uc001kwl.2   | uc001kwn.2   | Yes         |
|            |                                 | 104687375       |               |                       |                |                               |              |              |             |
| TMEM125    | Alternative 3' end              | chr1:43735698-  | NM_144626.2   | Induction of isoform 1 |                | Yes                           | uc001cir.2   |              | No          |
|            |                                 | 43736343        |               |                       |                |                               |              |              |             |
| CBWD2      | Alternative 3' end              | chr1:114195268- | NM_172003.3   | Induction of isoform 2 |                | Yes                           | uc002jju.2   | Not annotated| No          |
|            |                                 | 114253781       |               |                       |                |                               |              |              |             |
| NDUF3      | Alternative exon                | chr1:21:44313378-| NM_001001503.1| Switch to isoform 2 (exon excluded) | Yes | uc002zcm.2 | uc002zcn.2 | Yes |
|            |                                 | 44329773        |               |                       |                |                               |              |              |             |
| ZNF678     | Alternative exon                | chr1:227751220- | NM_178549.3   | Switch to isoform 2 (exon excluded) | Yes | uc009xet.1 | Not annotated | No |
|            |                                 | 227850164       |               |                       |                |                               |              |              |             |
| ZNF121     | Alternative exon                | chr19:9676404-  | NM_001308269.1| Switch to isoform 2 (exon excluded) | Yes | uc010xikq.1 | uc010xikp.1 | Yes |
|            |                                 | 9695209         |               |                       |                |                               |              |              |             |
| SPATC1L    | Alternative exon                | chr21:47581062- | NM_032261.4   | Induction of isoform 2 (exon included) | Yes | uc002zii.2 | Not annotated | No |
|            |                                 | 47604373        |               |                       |                |                               |              |              |             |
| MOCOS      | Alternative exon                | chr18:33767480- | NM_017947.2   | Switch to isoform 2 (exon excluded) | Yes | uc002kqg.3 | Not annotated | No |
|            |                                 | 33848685        |               |                       |                |                               |              |              |             |
| RBM45      | Alternative exon                | chr1:2:17897715-| NM_152945.3   | Switch to isoform 2 (exon included) | Yes | uc002ulv.2 | Not annotated | No |
|            |                                 | 178994382       |               |                       |                |                               |              |              |             |
| MIPEP      | Alternative exon                | chr13:24304328- | NM_006932.3   | Repression of isoform 2 (exon excluded) | Yes | uc001uox.3 | Not annotated | No |
|            |                                 | 24463587        |               |                       |                |                               |              |              |             |
| BBS4       | Alternative exon                | chr15:72978520- | NM_001320665.1| Induction of isoform 2 (exon included) | Yes | uc002avb.2 | Not annotated | No |
|            |                                 | 73030187        |               |                       |                |                               |              |              |             |
| FAM195A    | Alternative exon                | chr16:691804-  | NM_138418.3   | Switch to isoform 1 (exon excluded) | Yes | uc002cic.1 | uc002cie.2 | Yes |
|            |                                 | 6968474         |               |                       |                |                               |              |              |             |
| LINC01133  | Alternative exon                | chr1:159931008-| ENST0000043364.6| Induction of isoform 1 (exon excluded) | Both non- | Not annotated | uc001fuu.2 | No |
|            |                                 | 159948851       |               |                       | coding          |                               |              |              |             |
| SS18       | Alternative exon                | chr1:18:23596217-| NM_000107559.2| Switch to isoform 2 (exon excluded) | Yes | uc002kwn.2 | uc002kvn.2 | Yes |
|            |                                 | 23670611        |               |                       |                |                               |              |              |             |
| RHOC       | Alternative exon                | chr1:113343947-| ENST0000369636.8| Switch to isoform 2 (exon excluded) | No (5' UTR) | uc009wjk.1 | uc001ecr.1 | Yes |
|            |                                 | 113249757       |               |                       |                |                               |              |              |             |
| ZNF226     | Retained intron                | chr19:44669215-| NM_001319088.1| Switch to isoform 1 (intron included) | Yes | uc002oyo.2 | uc002oyn.2 | Yes |
|            |                                 | 44681838        |               |                       |                |                               |              |              |             |
Table 2. Quantitative changes in gene expression in response to androgens for the 73 genes with AR regulated alternative mRNA isoforms.

| LNCaP RNA-Seq (+/- androgens for 24 hours) | Reciprocal RNA-Seq (also change in 7 patients following ADT) |
|------------------------------------------|------------------------------------------------------------|
| No change | Upregulated | Downregulated | No change | Upregulated | Downregulated |
| RNL2       | LIG4        | NUP93         | LIG4      | TPD52       | None           |
| DENND1A    | TACC2       | PIK3R1        | TACC2     | AP2S1       |                |
| RAB3IL1    | RLN1        | MAPRE2        | NUP93     | DCXR        |                |
| OSBP1L1A   | AP2S1       | NDUFAF4       | RLN1      | PEX10       |                |
| TRIM16     | DCXR        | ACER3         | RLN2      | HMGCR       |                |
| Sep-05     | PEX10       | GPRIN2        | PIK3R1    | ALDH1A3     |                |
| RDH113     | SNAPC2      | TLE3          | MAPRE2    | FDT1        |                |
| ZFAND6     | ATP6V0D1    | TNRC6B        | NDUFAF4   | GREB1       |                |
| CDIP1      | ARRD1C1     | SORBS3        | SNAPC2    | NCAPD3      |                |
| LIMK2      | KLHL36      | ZNF121        | ATP6V0D1  | RAP1GAP     |                |
| TSC22D3    | VSG10L      | LINC01133     | ARRD1C1   | TMEM79      |                |
| GMFB       | HMGCR       | DENND1A       | KRT8      |             |                |
| MLST8      | CLK3        | KLHL36        | ELOVL1    |             |                |
| znf32      | RHN1        | RAB3IL1       | TMEM125   |             |                |
| C1QTNF3    | YIF1B       | ACER3         |          |             |                |
| UBE2D3     | PAK1IP1     | OSBP1L1A      |          |             |                |
| MAT2A      | ALDH1A3     | TRIM16        |          |             |                |
| CBWD2      | TRABD       | VSG10L        |          |             |                |
| ZNF678     | LIMCH1      | SEPT5         |          |             |                |
| MOCOS      | UBA1        | RDH13         |          |             |                |
|           | FDT1        | GPRIN2        |          |             |                |
|           | GREB1       | CLK3          |          |             |                |
|           | NCAPD3      | RHN1          |          |             |                |
|           | SLC36A4     | ZFAND6        |          |             |                |
|           | KLC2        | CDIP1         |          |             |                |
|           | RAP1GAP     | YIF1B         |          |             |                |
|           | TMEM79      | LIMK2         |          |             |                |
|           | NR4A1       | TSC22D3       |          |             |                |
|           | KRT8        | TRABD         |          |             |                |
|           | ELOVL1      | LIMCH1        |          |             |                |
|           | RCAN1       | GMFB          |          |             |                |
|           | CNNM2       | MLST8         |          |             |                |
|           | TMEM125     | TLE3          |          |             |                |
|           | NDUVF3      | UBA1          |          |             |                |
|           | SPATC1L     | TNRC6B        |          |             |                |
|           | RBM45       | SLC36A4       |          |             |                |
|           | MIPEP       | KLC2          |          |             |                |
|           | BBS4        | NR4A1         |          |             |                |
|           | FAM195A     | znf32         |          |             |                |
|           | SS18        | C1QTNF3       |          |             |                |
Of the 56 androgen regulated alternative promoters that were identified, 23 alternative promoters were induced by androgens (including LIG4, Figure 1B), 26 promoters were repressed by androgens, and for 7 genes there was a switch in usage from one promoter to another (Table 1). The alternative splicing events that were under androgen control included 12 alternative exons and one androgen-regulated intron retention (Table 1). 10 of these are novel to this study, including exclusion of an alternative exon in ZNF678 (Figure 1C). Of the alternative exons, six genes contained switches in previously unannotated protein-coding exons in response to androgen-exposure. We also identified four androgen regulated alternative mRNA 3’ end isoform switches, including a switch in the 3’ end of the mRNA transcript for the MAT2A gene (Figure 1D).

Androgen regulated events control the production of alternative protein isoforms, non-coding RNAs and alternative 5’ UTRs

48/73 (66%) of the androgen regulated alternative events detected in response to androgen stimulation are predicted to change the amino acid sequence of the resulting protein (Table 1). Some of these are already known to have a well characterised role in prostate cancer progression, including an alternative promoter in the oncogene TPD52 that produces a protein isoform called PrLZ (Figure 2A)46–49. Others are not so well characterised. Using western blotting we could detect a novel shorter protein isoform corresponding to androgen-driven selection of an alternative promoter in the TACC2 gene (Figure 2B); and exclusion of a cassette exon in the NDUFV3 gene, which we show also produces a novel shorter protein isoform (Figure 2C). We also detected a switch in the 3’ end of the mRNA transcript for the MAT2A gene, which is predicted to produce a protein isoform with a shorter C-terminal domain (Figure 1D); and induction of an alternative 3’ isoform of CNNM2, which is predicted to be missing a conserved CBS domain (Table 1 and Supplementary Figure 1).

11 of the remaining identified androgen-regulated alternative events change the expression of mRNAs from coding to non-coding or untranslated (not predicted to produce a protein) (Table 1). These included promoter switches for the RLN1 and RLN2 genes which encode peptide hormones that may be important in prostate cancer50–55. Androgens drive a promoter switch in both RLN1 and RLN2 to produce predicted non-coding or untranslated mRNA isoforms, reducing expression of protein-coding RLN1 and RLN2 mRNA isoforms. To
| Gene name                          | Function                                                                 | Clinical importance and roles in other cancer types                                                                 | Clinical importance and roles in prostate cancer |
|-----------------------------------|--------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|
| **TACC2** Transforming Acidic Coiled-Coil Containing Protein 2 | centrosome- and microtubule-interacting protein                          | Growth and prognosis of breast cancer<sup>56</sup>                                                                  | Castration-resistant growth of prostate cancer<sup>57</sup> |
| **LIG4**                          | DNA ligase with role in DNA repair                                       | Prognostic marker in nasopharyngeal cancer<sup>54</sup> Upregulated in colorectal cancer with role in wnt signalling<sup>49</sup> | Predictor of poor prognosis<sup>60</sup> |
| **RLN1 and RLN2** (Relaxin1 and 2) | Endocrine hormones (part of insulin gene superfamily)                   | Breast cancer invasiveness<sup>51,52</sup> Metastasis of human osteosarcoma<sup>53</sup> Thyroid cancer oncogenesis<sup>4,65</sup> | Well characterised role in the development and progression of prostate cancer<sup>70,71</sup> |
| **TPD52** (Tumor Protein D52)     | Role in proliferation and exo- and endocytic pathways                   | Well characterised role in numerous cancer types<sup>40,45-49</sup>                                                 | Known AR target, overexpressed and amplified in prostate cancer<sup>70</sup> Oncogene in prostate cancer<sup>71</sup> Neuroendocrine transdifferentiation of prostate cancer<sup>72</sup> Isoform produced by alternative promoter known as PrLZ and already linked to prostate cancer<sup>47-49,73-74</sup> |
| **FDFT1** (Farnesyl-Diphosphate Farnesyltransferase 1) | squalene synthase                                                      | Role in lung cancer metastasis<sup>75</sup>                                                                         | Linked to prostate cancer risk and aggressiveness<sup>76</sup> |
| **TLE3** (Transducin Like Enhancer Of Split 3) | Negative regulator of Wnt/β-catenin signaling                           | Predictive marker for response to therapy in ovarian and breast cancer<sup>77,78</sup> Represses colon cancer proliferation<sup>79</sup> | Upregulated in prostate tumours<sup>80</sup> and linked to wnt signalling in castrate resistant disease<sup>71</sup> |
| **CNNM2** (Cyclin & CBS Domain Divalent Metal Cation Transport Mediator 2) | Magnesium transporter                                                  | Proposed oncogenic role via increasing magnesium uptake<sup>82</sup>                                                | Unknown |
| **NUP93**                         | Nucleoprotein protein – role in apoptosis                              | Driver mutation linked to breast cancer<sup>83</sup>                                                              | Unknown |
| **MAT2A** Methionine adenosyltransferase II | Biosynthesis of S-adenosylmethionine, the principal biological methyl donor and precursor of polyamines and glutathione. | Upregulated in liver and colon cancer, potential drug target<sup>84,85</sup> Tumour suppressor in kidney carcinogenesis<sup>86</sup> Role in other cancer types<sup>87</sup> | Upregulated in prostate cancer and linked to cell migration via miR-34a and miR-34b<sup>87,88</sup> |
| **PIK3R1**                        | PI3K regulatory subunit                                                | Underexpressed in breast cancer<sup>89</sup> High mutation frequency in endometrial cancer<sup>90</sup>             | Controlled by androgens and repressed in prostate cancer cells<sup>11</sup> |
| **SNAPC2** (Small Nuclear RNA Activating Complex Polypeptide 2) | Subunit of the snRNA-activating protein complex. Necessary for RNA polymerase II and III dependent small-nuclear RNA gene transcription | Epigenetic silencing is prognostic in glioblastoma<sup>91</sup>                                                   | Unknown |
| **ZNF678** (Zinc Finger Protein 678) | Potential role in transcriptional regulation                           | Unknown                                                                                                              | Unknown |
| Gene name | Function | Clinical importance and roles in other cancer types | Clinical importance and roles in prostate cancer |
|-----------|----------|---------------------------------------------------|-----------------------------------------------|
| **NDUFV3** (NADH:Ubiquinone Oxidoreductase Subunit V3) | Subunit of part of the mitochondrial respiratory chain | Unknown | Androgen regulated alternative splice isoform previously identified by our exon array study<sup>39</sup> |
| **OSBPL1A** (Oxysterol Binding Protein Like 1A) | Intracellular lipid receptor | Alternative promoter use in colorectal cancer<sup>25</sup> | Unknown |
| **RDH13** (Retinol Dehydrogenase 13) | Role in retinoic acid production and protection against oxidative stress | Unknown | Unknown |
| **ZNF121** (Zinc Finger Protein 121) | Potential role in transcriptional regulation | Interacts with MYC. Upregulated in breast cancer<sup>31</sup> | Unknown |
| **SLC36A4.1** (Solute Carrier Family 36 Member 4) | Amino acid transporter | Unknown | Unknown |
| **RCAN1** (Regulator of Calcineurin 1) | Inhibits calcineurin-dependent signaling pathways | Inhibits NF-κB and suppresses lymphoma growth in mice<sup>26</sup>. Role in cancer cell migration<sup>26</sup> | Unknown |
| **DCXR** (Dicarbonyl & l-xylulose reductase) | Role in the uronate cycle of glucose metabolism | Low expression indicates poor prognosis for hepatocellular carcinoma<sup>26</sup>. Role in cell adhesion<sup>27,28</sup> | Upregulated and potential biomarker in prostate cancer<sup>29</sup> |
| **NDUFAF4** (NADH:Ubiquinone Oxidoreductase Complex Assembly Factor 4) | Role in the mitochondrial respiratory chain | Unknown | Unknown |
| **MAPRE2** (Microtubule Associated Protein RP/EB Family Member 2) | Microtubule-associated protein that is necessary for spindle symmetry during mitosis | Role in the invasion of pancreatic cancer cells<sup>100</sup> | Unknown |
| **PEX10** (Peroxisomal Biogenesis Factor 10) | Involved in import of peroxisomal matrix proteins | Unknown | Unknown |
| **AP2S1** (Adaptor Related Protein Complex 2 Sigma 1 Subunit) | Function in protein transport across membranes | Unknown | Unknown |
| **LINC01133** (long non-coding RNA) | Long non-coding RNA | Poor prognosis in colorectal cancer<sup>101</sup>. Upregulated and linked to poor prognosis in lung cancer<sup>102</sup> | Unknown |
| **ZNF226** (Zinc Finger Protein 226) | Potential role in transcriptional regulation | Unknown | Unknown |
| **CDIP1** (Cell death inducing p53 target 1) | p53 apoptotic effector | Regulates TNF-alpha-mediated apoptosis | Sensitivity to TNFα-induced apoptosis in cancer cells<sup>31</sup> | Unknown |
Figure 1. Global identification of androgen-dependent mRNA isoform production in prostate cancer cells predicts a major role for alternative promoter utilisation. (A) Analysis of RNAseq data from LNCaP cells grown with (A+) or without androgens (R1881) (steroid deplete, SD) for 24 hours identified 73 androgen regulated alternative mRNA isoforms. The 73 alternative events were generated via androgen-regulated utilisation of 56 alternative promoters, 4 alternative 3’ ends and 13 alternative splicing events. (B) Androgens drive a promoter switch in the LIG4 gene, which produces an mRNA isoform with an alternative 5’UTR. Visualisation of our LNCaP cell RNA-seq reads for the LIG4 gene on the UCSC genome browser identified a switch from promoter 1 to alternative promoter 2 in cells grown in the presence of androgens. Promoter 2 is predicted to produce a different 5’UTR without influencing the protein sequence (left panel). Quantitative PCR using primers specific to each promoter indicate that in response to androgens there is repression of promoter 1 and induction of promoter 2 (right panel). (C) Androgens drive alternative splicing of the ZNF678 gene. Visualisation of our LNCaP cell RNA-seq reads for the ZNF678 gene on the UCSC genome browser identified a switch to inclusion of a cassette exon in the presence of androgens. Inclusion of the alternative cassette exon in the ZNF678 gene is predicted to induce a switch to an alternative non-coding mRNA isoform (left panel). Quantitative PCR using primers in flanking exons confirmed increased inclusion of the alternative exon in LNCaP cells exposed to androgens (right panel). (D) Androgens promote selection of an alternative 3’ end for the MAT2A gene. Visualisation of our LNCaP cell RNA-seq reads for the MAT2A gene on the UCSC genome browser indicates a switch to reduced usage of an alternative 3’ end in the presence of androgens (left panel). Quantitative PCR using primers specific to each isoform confirmed down-regulation of an alternative 3’ end (p<0.01). Alternative 3’ ends for the MAT2A gene are predicted to produce proteins with different amino acid sequences and to influence a known Pfam domain (right panel).
Figure 2. Androgen regulated mRNA isoform switches control alternative protein isoforms and non-coding RNAs. (A) Androgens induce an alternative promoter in the oncogene *TPDS2* that produces an isoform called PrLZ. Visualisation of our LNCaP cell RNA-seq reads for the *TPDS2* gene on the UCSC genome browser identified a switch from promoter 1 to alternative promoter 2 in cells grown in the presence of androgens. Promoter 2 is known to produce an alternative protein isoform of *TPDS2* known as PrLZ (left panel). Quantitative PCR using primers specific to each promoter indicate an induction of the PrLZ isoform in response to androgens (middle panel). PrLZ has an alternative N-terminal amino acid sequence which results in an alternative protein isoform and disrupts a known Pfam domain (right panel). (B) Androgens induce an alternative promoter in the TACC2 gene that produces a novel alternative protein isoform. Visualisation of our LNCaP cell RNA-seq reads for the TACC2 gene on the UCSC genome browser identified a switch from promoter 1 to alternative promoter 2 in cells grown in the presence of androgens. Promoter 2 is predicted to produce an alternative shorter protein isoform of TACC2 (isoform 2) (left panel). Quantitative PCR using primers specific to each promoter indicate a switch from isoform 1 to isoform 2 in response to androgens (middle panel). Detection of TACC2 protein in LNCaP by western blotting (cells were grown with or without androgens for 24 or 48 hours). Tubulin was used as a loading control. Exposure to androgens for 48 hours induces expression of the alternative TACC2 protein isoform (right panel). (C) Androgens drive alternative splicing of the *NDUFV3* gene. Visualisation of our LNCaP cell RNA-seq reads for the *NDUFV3* gene on the UCSC genome browser identified a switch to exclusion of a cassette exon in the presence of androgens (left panel). Quantitative PCR using primers in flanking exons confirmed less inclusion of the alternative exon in LNCaP cells exposed to androgens (middle panel). Exclusion of the alternative cassette exon is predicted to produce an alternative protein isoform. Detection of NDUFV3 protein in LNCaP cells using western blotting (right panel). (D) Androgens suppress an alternative promoter in the *RLN2* gene, which produces a shorter non-coding mRNA isoform. Visualisation of our LNCaP cell RNA-seq reads for the *RLN2* gene on the UCSC genome browser identified a switch from promoter 1 to alternative promoter 2 in cells grown in the presence of androgens. Promoter 2 is predicted to produce an untranslatable non-coding mRNA isoform (left panel). Quantitative PCR using primers specific to each promoter indicated a significant switch in promoter usage in response to androgens (middle panel). Detection of RLN2 protein in LNCaP by western blotting (cells were grown with or without androgens for 48 hours). Actin was used as a loading control. As seen previously, androgens suppress RLN2 protein levels.

test whether prostate cancer cells turn off gene expression by switching between utilisation of promoters that generate coding and noncoding mRNAs, we analysed RLN2 protein levels. Consistent with our hypothesis and a previous study, RLN2 protein production was negatively regulated by androgens in parallel to the switch to the non-coding mRNA isoform (Figure 2D).

14 of the identified androgen-dependent mRNA isoforms lead to result in coding mRNAs with altered 5′ untranslated regions (5′ UTR) with no impact on the coding sequence. These include a promoter switch in the *LIG4* gene (Figure 1B).

Differential expression of androgen-dependent mRNA isoforms in prostate adenocarcinoma versus normal tissue

To investigate potential links between androgen-dependent mRNA isoforms and tumourigenesis, we analysed the expression of 41 androgen-regulated mRNA isoforms in clinical prostate adenocarcinoma and normal prostate tissues. This analysis utilised transcriptomic data from 497 tumour samples and 52 normal samples in the PRAD TCGA cohort. The remaining isoform pairs identified within our dataset have not been previously annotated by UCSC, therefore it was not possible to include them in our comparison. A description of the cohort used is summarised in Table 4.

33 of the 42 mRNA isoform pairs exhibited significant differences in the expression of at least one of the isoforms, or in the isoform expression ratio between tumour and normal tissues (Table 5). 13 of those tumour-specific alterations mimicked the effect of androgen stimulation in LNCaP cells; the changes were in form of alternative promoters for TACC2, TPDS2, NUP93, PIK3R1, RDH13, ZFAND6, CDIP1, YIF1B, LIMP2, and FDFT1; an alternative 3′ end in CNNM2; and alternative exons in

| Features | Total Cases |
|----------|-------------|
| Cohort   | 497 patients |
| Tumour   | 497         |
| Normal   | 52 (w/tumour matched sample available) |
| Gleason grade |
| 6        | 50          |
| 7        | 287         |
| 8        | 67          |
| 9        | 140         |
| 10       | 4           |
| Tumour stage |
| T2a      | 14          |
| T2b      | 10          |
| T2c      | 192         |
| T3a      | 173         |
| T3b      | 140         |
| T4       | 12          |
| Gleason grade (alternative gleason grade grouping) |
| 1 (primary + secondary score ≤ 6) | 50 |
| 2 (3 + 4) | 171         |
| 3 (4 + 3) | 123         |
| 4 (4 + 4) | 93          |
| 5 (primary + secondary score ≥ 9) | 111          |

All tumours were hormone naive (not subject to ADT) at the time of sample collection.
| Gene   | Event type            | Change with androgens (LNCaP) | log2FC | Av. Exp. (TPM) | PSI   | Consistency of change in tumours |
|--------|-----------------------|-------------------------------|--------|----------------|-------|---------------------------------|
| LIG4   | Alternative promoter  | Induction of promoter 2       | -0.81  | 4.31E-02       | 1.28  | Opposite                        |
| TAC2   | Alternative promoter  | Induction of promoter 2       | 0.90   | 2.22           | 0.16  | Consistent                      |
| TDO2   | Alternative promoter  | Induction of promoter 2       | -0.34  | 0.17           | 1.87  | Consistent                      |
| NUP93  | Alternative promoter  | Induction of promoter 1       | 0.25   | 39.20          | 0.31  | Consistent                      |
| RLN1   | Alternative promoter  | Induction of promoter 2       | -0.45  | 133.50         | 0.06  | Consistent                      |
| AP2S1  | Alternative promoter  | Induction of promoter 2       | 0.48   | 22.24E-05      | --    | Not assessed                    |
| RLN2   | Alternative promoter  | Induction of promoter 1       | 0.48   | 191.44         | --    | Not assessed                    |
| PK3R1  | Alternative promoter  | Induction of promoter 2       | -1.79  | 1.75           | -1.79  | Opposite                        |
| MAPRE2 | Alternative promoter  | Switch to promoter 2          | 0.48   | 33.90          | 0.31  | Consistent                      |
| NDUFAF1| Alternative promoter  | Switch to promoter 2          | 0.48   | 1.52           | 0.34  | Inconclusive                    |
| DCRR   | Alternative promoter  | Repression of promoter 1      | 0.92   | 7.15           | 1.26  | Consistent                      |
| PEX10  | Alternative promoter  | Repression of promoter 1      | 0.92   | 75.56          | 1.26  | Consistent                      |
| SNAPIPC2| Alternative promoter | Repression of promoter 1      | 0.38   | 0.12           | 1.26  | Consistent                      |
| ATPLF0D1| Alternative promoter | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| ARD1C  | Alternative promoter  | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| DENND1A| Alternative promoter  | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| KLI1   | Alternative promoter  | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| RAB8A1 | Alternative promoter  | Repression of promoter 1      | 0.34   | 0.34           | 0.12  | Consistent                      |
| ACP3   | Alternative promoter  | Repression of promoter 1      | 0.92   | 0.92           | 0.12  | Consistent                      |
| CSRPLA | Alternative promoter  | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| TRIM16 | Alternative promoter  | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| VAG1L  | Alternative promoter  | Repression of promoter 1      | 0.46   | 0.46           | 0.12  | Consistent                      |
| Gene    | SEPT5 | HMGCR | RDH13 | GRIN2 | CL3K | RHH1 | ZFAND6 | CDP1 | YIF1B | LIMK2 | TSC2D3 | ALDH1A3 | LIMCH1 | TFABD | GABP | GMFB | F1000Research 2018, 7:1189 Last updated: 18 SEP 2018 |
|---------|-------|-------|-------|-------|------|------|--------|------|-------|-------|--------|---------|--------|-------|------|-----|-------|
| PSI     | 3.86  | 1.09  | 1.47  | 1.47  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Av. PSI | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Consistency of change in Av. PSI | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite |
| FDR    | 0.80  | 0.80  | 0.80  | 0.80  | 0.80 | 0.80 | 0.80   | 0.80 | 0.80  | 0.80  | 0.80   | 0.80    | 0.80   | 0.80  | 0.80 | 0.80 | 0.80  |
| PSI     | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Av. PSI | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Consistency of change in Av. PSI | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite |
| FDR    | 0.80  | 0.80  | 0.80  | 0.80  | 0.80 | 0.80 | 0.80   | 0.80 | 0.80  | 0.80  | 0.80   | 0.80    | 0.80   | 0.80  | 0.80 | 0.80 | 0.80  |
| PSI     | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Av. PSI | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Consistency of change in Av. PSI | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite |
| FDR    | 0.80  | 0.80  | 0.80  | 0.80  | 0.80 | 0.80 | 0.80   | 0.80 | 0.80  | 0.80  | 0.80   | 0.80    | 0.80   | 0.80  | 0.80 | 0.80 | 0.80  |
| PSI     | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Av. PSI | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Consistency of change in Av. PSI | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite |
| FDR    | 0.80  | 0.80  | 0.80  | 0.80  | 0.80 | 0.80 | 0.80   | 0.80 | 0.80  | 0.80  | 0.80   | 0.80    | 0.80   | 0.80  | 0.80 | 0.80 | 0.80  |
| PSI     | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Av. PSI | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Consistency of change in Av. PSI | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite |
| FDR    | 0.80  | 0.80  | 0.80  | 0.80  | 0.80 | 0.80 | 0.80   | 0.80 | 0.80  | 0.80  | 0.80   | 0.80    | 0.80   | 0.80  | 0.80 | 0.80 | 0.80  |
| PSI     | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Av. PSI | 3.86  | 1.09  | 1.30  | 1.30  | 1.30 | 1.30 | 1.30   | 1.30 | 1.30  | 1.30  | 1.30   | 1.30    | 1.30   | 1.30  | 1.30 | 1.30 | 1.30  |
| Consistency of change in Av. PSI | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite | Opposite |
| FDR    | 0.80  | 0.80  | 0.80  | 0.80  | 0.80 | 0.80 | 0.80   | 0.80 | 0.80  | 0.80  | 0.80   | 0.80    | 0.80   | 0.80  | 0.80 | 0.80 | 0.80  |
| Gene     | Event type          | Change with androgens (LNCap) | log2FC  | FDR   | Consistency of change in tumours |
|----------|---------------------|-------------------------------|---------|-------|----------------------------------|
| C1QTNF3  | Alternative promoter | Induction of promoter 1       | 0.30    | 0.50  | --                               |
| UBE2D3   | Alternative promoter | Switch to promoter 2          | -0.50   | -0.13 | --                               |
| KRT8     | Alternative promoter | Repression of promoter 1      | -0.10   | 0.10  | --                               |
| ELOVL1   | Alternative promoter | Repression of isoform 1       | -0.36   | -0.21 | --                               |
| SOX9     | Alternative promoter | Induction of isoform 2        | 0.21    | 0.21  | --                               |
| CACNA1D2 | Alternative promoter | Induction of isoform 1        | 0.00    | 0.00  | --                               |
| CACNA1D2 | Alternative promoter | Switch to isoform 2           | 0.00    | 0.00  | --                               |
| NDUFA3   | Alternative exon    | Repression of isoform 1       | 0.00    | 0.00  | --                               |
| ZNF121   | Alternative exon    | Switch to isoform 2 (exon 1)  | 0.00    | 0.00  | --                               |
| SPAC5L   | Alternative exon    | Repression of isoform 1 (exon| 0.00    | 0.00  | --                               |
| ZNF226   | Alternative exon    | Switch to isoform 1 (exon 1)  | 0.00    | 0.00  | --                               |
| MIPEP    | Alternative exon    | Switch to isoform 1 (exon 1)  | 0.00    | 0.00  | --                               |
| BBS4     | Alternative promoter | Switch to isoform 2 (exon 2)  | 0.00    | 0.00  | --                               |
| FAM19A   | Alternative promoter | Switch to isoform 1 (exon 2)  | 0.00    | 0.00  | --                               |
| LINC01133| Alternative promoter | Switch to isoform 1 (exon 2)  | 0.00    | 0.00  | --                               |
| SS18     | Alternative promoter | Switch to isoform 1 (exon 2)  | 0.00    | 0.00  | --                               |
| RHOC     | Alternative promoter | Switch to isoform 1 (exon 2)  | 0.00    | 0.00  | --                               |
| ZNF226   | Alternative promoter | Switch to isoform 1 (exon 2)  | 0.00    | 0.00  | --                               |

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NDUFS3 and SS18 (Figure 3, Table 5 & Supplementary Figure 2). Two of the alternative promoters (ZFAND6 and CDIP1) are predicted to introduce a change in the 5'UTR, whereas all the others are predicted to alter the resulting protein isoform. A number of mRNA isoforms that were androgen responsive in LNCaP cells showed tumour specific alterations opposite to the effect of androgen stimulation. These were LIG4, MAPRE2, OSBPL1A, SEPT5, NRE41, and RCAN1 (all predicted to alter the resulting protein isoform except LIG4). For the remaining 14 mRNA isoform pairs, the data was inconclusive according to the consistency conditions listed in the methods section (Table 5).

Changes in androgen-dependent mRNA isoform expression during tumour progression

We next investigated whether the identified androgen-dependent mRNA isoforms are differentially expressed during prostate cancer progression by correlating the expression levels of each isoform with Gleason scores and prostate tumour grades within the PRAD TCGA cohort (Figure 4 & Figure 5, Table 6 & Table 7 and Supplementary Figure 3 & Supplementary Figure 4). For 6 of the alternative mRNA isoforms responsive to androgens (made from alternative promoters in LIG4, OSBPL1A, CLK3, TSC22D3 & ZNF32 and utilising an alternative exon in ZNF121), the expression changed significantly with Gleason score and showed specific alterations consistent with the effect of androgen stimulation. Conversely, 9 alternative isoforms (which were androgen responsive in LNCaP cells) showed tumour specific alterations opposite to the effect of androgen stimulation (including an alternative promoters in NUP93 and the alternative 3’ end of MAT2A). 3 androgen regulated mRNA isoforms (OSBPL1A, CLK3 and TSC22D3) change significantly with both Gleason grade and tumour stage.

Androgen exposure further drives a smaller number of alternative splicing events suggesting that the AR could contribute to altered patterns of splicing in prostate cancer cells. Tumour progression is believed to be associated with a coordinated change in splicing patterns which is regulated by several factors including signalling molecules1. We also identified 4 AR regulated alternative mRNA 3’ end isoform switches. This is the first time that regulation of 3’ mRNA end processing has been shown to be controlled by androgens. The selection of alternative 3’ ends can produce mRNA isoforms differing in the length of their 3’ UTRs (which can lead to the inclusion or exclusion of regulatory elements and influence gene expression), or in their C-terminal coding region (which can contribute to proteome diversity). Defective 3’ mRNA processing of numerous genes has been linked to an oncogenic phenotype15-19 and the 3’ mRNA end profiles of samples from multiple cancer types significantly differ from those of healthy tissue samples15,19-21.

Based on the findings presented in this study, we propose that activated AR has the ability to coordinate both transcriptional activity and mRNA isoform decisions through the recruitment of co-regulators to specific promoters. The genomic action of the AR is influenced by a large number of collaborating transcription factors22-24. Specifically, Sam68 and p68 have been shown to modulate AR dependent alternative splicing of specific genes and are significantly overexpressed in prostate cancer21,22. In future work it will be important to define the role of specific AR co-regulators in AR mediated isoform selection.

Some of the androgen dependent mRNA isoforms identified here are predicted to yield protein isoforms that may be clinically important, or to switch off protein production via generation of noncoding mRNA isoforms. Although the functional significance of the alternative mRNA isoforms identified in this study is yet largely unexplored, as is their role in the cellular response to androgens, the presented results emphasize the importance of analysing gene regulation and function at the mRNA isoform level.
Figure 3. Differential expression of androgen dependent mRNA isoforms in prostate cancer versus normal tissue within the PRAD TCGA cohort for TPDS2, TACC2, NDUVF3 and CNNM2. Violin-boxplots of expression in transcripts per million mapped reads (TPM) of isoforms 1 (left panel) and 2 (central panel), and of their expression ratio in PSI (right panel) in normal and tumour samples. The mean log2 fold-change (logFC) in expression between tumour and normal samples and the associated FDR-adjusted p-value for the moderated t-statistic of differential expression are shown for both isoforms (left and central panels). The mean difference in PSI (deltaPSI) between tumour and normal samples and the associated FDR-adjusted p-value for the Mann-Whitney U test of differential splicing are shown (right panel).
Figure 4. Differential alternative mRNA isoform expression in the TGCA PRAD cohort across different Gleason grades for OSBPL1A, CLK3, TSC22D and ZNF121. Violin-boxplots of expression in transcripts per million mapped reads (TPM) of Isoforms 1 (left panel) and 2 (central panel), and of their expression ratio (right panel) by Gleason grade. Their respective Spearman's correlation coefficient (Rho) with grade and associated FDR-adjusted p-value are shown.
Figure 5. Differential alternative mRNA isoform expression in the TGCA PRAD cohort across different tumour stages for OSBPL1A, CLK3 and TSC22D3. Violin-boxplots of expression in transcripts per million mapped reads (TPM) of Isoforms 1 (left panel) and 2 (central panel), and of their expression ratio (right panel) by tumour stage. Their respective Spearman’s correlation coefficient (Rho) with stage and associated FDR-adjusted p-value are shown.
Table 6. Summarised results of the correlation analysis of androgen-regulated isoforms expression with Gleason score in the TCGA PRAD cohort.

| Gene       | Event type | Change with androgens (LNCap) | Isoform 1 |   |   |   |   | Consistency of change with Gleason |
|------------|------------|-------------------------------|-----------|---|---|---|---|-----------------------------------|
| LIG4       | Alternative promoter | Induction of promoter 2 | 0.07     | 1.92E-01 | 0.09 | 1.07E-01 | -0.18 | 4.21E-04 | Consistent - |
| TACC2      | Alternative promoter | Repression of promoter 1 | -0.08    | 1.55E-01 | 0.01 | 9.26E-01 | -0.08 | 1.88E-01 | Inconclusive |
| TP5D2      | Alternative promoter | Induction of promoter 2 | 0.00     | 9.51E-01 | 0.02 | 7.73E-01 | 0.00  | 9.46E-01 | Inconclusive |
| NUP93      | Alternative promoter | Induction of promoter 1 | -0.18    | 7.92E-04 | -0.07 | 1.81E-01 | 0.04  | 4.75E-01 | Opposite |
| RLN1       | Alternative promoter | Repression of promoter 2 | -0.16    | 1.98E-03 | --   | --   | --   | --   | Not assessed |
| AP2S1      | Alternative promoter | Induction of promoter 2 | -0.01    | 8.72E-01 | --   | --   | --   | --   | Not assessed |
| RLN2       | Alternative promoter | Induction of promoter 1 | -0.10    | 6.03E-02 | --   | --   | --   | --   | Not assessed |
| PIK3R1     | Alternative promoter | Repression of promoter 2 | -0.07    | 2.51E-01 | 0.09 | 1.20E-01 | -0.17 | 1.29E-03 | Inconclusive |
| MAPRE2     | Alternative promoter | Switch to promoter 2 | -0.07    | 1.92E-01 | -0.06 | 2.73E-01 | 0.06  | 3.23E-01 | Inconclusive |
| NDUFAF4    | Alternative promoter | Repression of promoter 2 | 0.00     | 9.79E-01 | --   | --   | --   | --   | Not assessed |
| DCXR       | Alternative promoter | Repression of promoter 2 | -0.29    | 4.07E-09 | --   | --   | --   | --   | Not assessed |
| PEX10      | Alternative promoter | Switch to promoter 2 | 0.08     | 1.50E-01 | --   | --   | --   | --   | Not assessed |
| SNAC2C     | Alternative promoter | Switch to promoter 2 | 0.15     | 5.48E-03 | -0.18 | 3.55E-04 | 0.21  | 5.13E-05 | Opposite |
| ATP6V0D1   | Alternative promoter | Repression of promoter 2 | -0.11    | 3.43E-02 | --   | --   | --   | --   | Not assessed |
| ARRD1C     | Alternative promoter | Induction of promoter 2 | 0.12     | 2.00E-02 | --   | --   | --   | --   | Not assessed |
| DENND1A    | Alternative promoter | Repression of promoter 2 | -0.02    | 8.10E-01 | --   | --   | --   | --   | Not assessed |
| KHL3L       | Alternative promoter | Induction of promoter 2 | -0.13    | 1.67E-02 | --   | --   | --   | --   | Not assessed |
| RAB3L1     | Alternative promoter | Repression of promoter 2 | 0.06     | 3.17E-01 | 0.32 | 9.13E-12 | -0.02 | 7.15E-01 | Opposite |
| ACER3      | Alternative promoter | Repression of promoter 2 | 0.16     | 3.79E-03 | --   | --   | --   | --   | Not assessed |
| OSBP1A     | Alternative promoter | Induction of promoter 2 | 0.05     | 4.00E-01 | 0.13 | 1.58E-02 | -0.07 | 2.33E-01 | Consistent |
| TRIM16     | Alternative promoter | Induction of promoter 2 | 0.10     | 6.06E-02 | --   | --   | --   | --   | Not assessed |
| VSIG10L    | Alternative promoter | Induction of promoter 1 | -0.16    | 1.98E-03 | --   | --   | --   | --   | Not assessed |
| SEPT5      | Alternative promoter | Repression of promoter 2 | 0.17     | 1.12E-03 | 0.12 | 1.93E-02 | -0.04 | 4.91E-01 | Opposite |
| HMGCR      | Alternative promoter | Repression of promoter 1 | 0.03     | 6.56E-01 | -0.05 | 4.54E-01 | 0.07  | 2.33E-01 | Inconclusive |
| RDH13      | Alternative promoter | Induction of promoter 1 | 0.03     | 7.01E-01 | 0.08 | 1.20E-01 | -0.10 | 1.00E-01 | Inconclusive |
| GPRIN2     | Alternative promoter | Repression of promoter 2 | --       | --       | -0.01 | 8.93E-01 | --   | --   | Not assessed |
| CLK3       | Alternative promoter | Repression of promoter 1 | -0.13    | 1.58E-02 | -0.05 | 3.98E-01 | 0.07  | 2.33E-01 | Consistent |
| RNH1       | Alternative promoter | Induction of promoter 1 | 0.05     | 4.41E-01 | 0.07 | 1.83E-01 | -0.01 | 9.23E-01 | Inconclusive |
| ZFAND6     | Alternative promoter | Repression of promoter 2 | 0.07     | 1.87E-01 | 0.05 | 3.82E-01 | -0.03 | 6.36E-01 | Inconclusive |
| CDIP1      | Alternative promoter | Repression of promoter 2 | 0.02     | 8.10E-01 | 0.03 | 6.81E-01 | -0.01 | 9.23E-01 | Inconclusive |
| YIF1B      | Alternative promoter | Switch to promoter 2 | 0.02     | 8.10E-01 | -0.04 | 5.42E-01 | 0.05  | 4.39E-01 | Inconclusive |
| LIMK2      | Alternative promoter | Switch to promoter 2 | -0.02    | 8.10E-01 | -0.03 | 6.30E-01 | 0.00  | 9.49E-01 | Inconclusive |
| TSC22D3    | Alternative promoter | Repression of promoter 1 | -0.15    | 5.15E-03 | -0.01 | 9.26E-01 | -0.09 | 1.14E-01 | Consistent |
| ALDH1A3    | Alternative promoter | Repression of promoter 1 | -0.12    | 2.00E-02 | --   | --   | --   | --   | Not assessed |
| TRABD      | Alternative promoter | Switch to promoter 2 | 0.14     | 8.04E-03 | -0.04 | 5.43E-01 | 0.05  | 4.39E-01 | Inconclusive |
| LIMCH1     | Alternative promoter | Repression of promoter 2 | 0.05     | 4.34E-01 | --   | --   | --   | --   | Not assessed |
| GMFB       | Alternative promoter | Induction of promoter 2 | 0.08     | 1.55E-01 | --   | --   | --   | --   | Not assessed |
| MLST8      | Alternative promoter | Switch to promoter 1 | 0.19     | 5.32E-04 | 0.19  | 2.05E-04 | 0.07  | 2.14E-01 | Inconclusive |
| TLE3       | Alternative promoter | Induction of promoter 2 | 0.05     | 4.28E-01 | -0.10 | 7.19E-02 | 0.07  | 2.33E-01 | Inconclusive |
| Gene     | Event type       | Change with androgens (LNCap) | Isoform 1 | Isoform 2 | PSI          | Consistency of change with Gleason |
|----------|------------------|-------------------------------|-----------|-----------|--------------|-----------------------------------|
| UBA1     | Alternative promoter | Repression of promoter 1     | 0.09      | 8.99E-02  | 0.03         | 5.95E-01                          | 0.01         | 8.68E-01 | Inconclusive |
| TNRC6B   | Alternative promoter | Repression of promoter 2     | -0.05     | 4.00E-01  | -0.09        | 1.19E-01                          | 0.09         | 1.11E-01 | Inconclusive |
| FDF1     | Alternative promoter | Repression of promoter 2     | -0.02     | 7.41E-01  | 0.07         | 2.07E-01                          | -0.07        | 2.14E-01 | Inconclusive |
| GREB1    | Alternative promoter | Induction of promoter 2      | -0.05     | 4.41E-01  | -0.14        | 5.45E-03                          | 0.04         | 4.60E-01 | Opposite   |
| NCAPO3   | Alternative promoter | Induction of promoter 2      | -0.23     | 3.61E-06  | --           | --                                | --           | --        | Not assessed |
| SLC36A4  | Alternative promoter | Induction of promoter 2      | 0.12      | 1.88E-02  | --           | --                                | --           | --        | Not assessed |
| KLC2     | Alternative promoter | Repression of promoter 1     | -0.02     | 8.10E-01  | 0.13         | 1.58E-02                          | -0.04        | 4.60E-01 | Inconclusive |
| RAP1GAP  | Alternative promoter | Repression of promoter 1     | 0.01      | 8.79E-01  | --           | --                                | --           | --        | Not assessed |
| TMEM79   | Alternative promoter | Repression of promoter 1     | -0.04     | 4.70E-01  | 0.15         | 3.46E-03                          | -0.09        | 1.11E-01 | Inconclusive |
| NR4A1    | Alternative promoter | Induction of promoter 2      | 0.10      | 5.44E-02  | 0.00         | 9.79E-01                          | 0.10         | 7.40E-02 | Inconclusive |
| ZNF32    | Alternative promoter | Repression of promoter 2     | -0.22     | 1.32E-05  | -0.22        | 1.11E-05                          | -0.09        | 1.31E-01 | Consistent |
| C1QTNF3  | Alternative promoter | Induction of promoter 1      | 0.08      | 1.58E-01  | --           | --                                | --           | --        | Not assessed |
| UBE2D3   | Alternative promoter | Switch to promoter 2         | 0.18      | 7.24E-04  | 0.08         | 1.27E-01                          | -0.02        | 7.15E-01 | Inconclusive |
| KRT8     | Alternative promoter | Repression of promoter 1     | -0.05     | 3.81E-01  | -0.16        | 2.07E-03                          | 0.01         | 8.68E-01 | Inconclusive |
| ELOVL1   | Alternative promoter | Induction of promoter 2      | 0.18      | 7.24E-04  | --           | --                                | --           | --        | Not assessed |
| RCAN1    | Alternative promoter | Induction of promoter 2      | 0.10      | 5.31E-02  | -0.01        | 8.70E-01                          | 0.12         | 3.69E-02 | Inconclusive |
| SORBS3   | Alternative promoter | Induction of promoter 2      | 0.12      | 2.21E-02  | --           | --                                | --           | --        | Not assessed |
| MAT2A    | Alternative 3' end | Repression of isoform 2      | 0.04      | 5.39E-01  | 0.27         | 3.68E-08                          | -0.33        | 8.82E-13 | Opposite   |
| CNNM2    | Alternative 3' end | Induction of isoform 1       | -0.06     | 3.30E-01  | 0.03         | 5.87E-01                          | -0.08        | 2.04E-01 | Inconclusive |
| TMEM125  | Alternative 3' end | Induction of isoform 1       | --        | --        | -0.19        | 2.05E-04                          | --           | --        | Not assessed |
| CBWD2    | Alternative 3' end | Induction of isoform 2       | 0.13      | 1.37E-02  | --           | --                                | --           | --        | Not assessed |
| NDUV3    | Alternative exon   | Switch to isoform 2 (exon excluded) | 0.14 | 8.04E-03 | -0.07 | 2.48E-01 | 0.13 | 2.23E-02 | Opposite |
| ZNF678   | Alternative exon   | Switch to isoform 2 (exon excluded) | -0.07 | 1.87E-01 | -- | -- | -- | -- | Not assessed |
| ZNF121   | Alternative exon   | Switch to isoform 2 (exon excluded) | -0.13 | 1.63E-02 | 0.08 | 1.20E-01 | -0.14 | 1.27E-02 | Consistent |
| SPATC1L  | Alternative exon   | Induction of isoform 2 (exon included) | -0.13 | 1.58E-02 | -- | -- | -- | -- | Not assessed |
| MOCOS    | Alternative exon   | Switch to isoform 2 (exon included) | -0.01 | 8.72E-01 | -- | -- | -- | -- | Not assessed |
| RBM45    | Alternative exon   | Switch to isoform 2 (exon included) | 0.12 | 2.45E-02 | -- | -- | -- | -- | Not assessed |
| MIPEP    | Alternative exon   | Repression of isoform 2 (exon included) | -0.14 | 9.92E-03 | -- | -- | -- | -- | Not assessed |
| BBS4     | Alternative exon   | Induction of isoform 2 (exon included) | -0.08 | 1.87E-01 | -- | -- | -- | -- | Not assessed |
| FAM195A  | Alternative exon   | Switch to isoform 1 (exon included) | 0.04 | 5.43E-01 | 0.14 | 5.35E-03 | -0.18 | 4.65E-04 | Opposite |
| LINC01133| Alternative exon   | Induction of isoform 1 (exon included) | -- | -- | -0.02 | 7.51E-01 | -- | -- | Not assessed |
| SS18     | Alternative exon   | Switch to isoform 2 (exon included) | 0.04 | 4.86E-01 | -0.06 | 2.51E-01 | 0.07 | 2.33E-01 | Inconclusive |
| RHOC     | Alternative exon   | Switch to isoform 2 (exon included) | 0.29 | 4.07E-09 | 0.15 | 4.24E-03 | 0.21 | 3.63E-05 | Opposite |
| ZNF226   | Retained intron    | Switch to isoform 1 (intron included) | 0.01 | 8.67E-01 | -0.10 | 7.49E-02 | 0.11 | 6.74E-02 | Inconclusive |
Table 7. Summarised results of the correlation analysis of androgen-regulated isoforms expression with tumour stage in the TCGA PRAD cohort (related to Figure 4 and Supplementary Figure 5).

| Gene   | Event type               | Change with androgens (LNCap) | Isoform 1 Rho | FDR | Isoform 2 Rho | FDR | PSI Consistency | Consistency of change with stage |
|--------|--------------------------|------------------------------|----------------|-----|----------------|-----|----------------|----------------------------------|
| LIG4   | Alternative promoter     | Induction of promoter 2      | -0.04          | 6.05E-01 | 0.02           | 6.82E-01 | -0.09          | 1.82E-01                         | Inconclusive                     |
| TACC2  | Alternative promoter     | Repression of promoter 1     | -0.08          | 1.74E-01 | -0.05          | 4.47E-01 | -0.04          | 5.65E-01                         | Inconclusive                     |
| TPD52  | Alternative promoter     | Induction of promoter 2      | -0.02          | 7.85E-01 | -0.02          | 6.82E-01 | -0.02          | 7.95E-01                         | Inconclusive                     |
| NUP93  | Alternative promoter     | Induction of promoter 1      | -0.12          | 3.95E-02 | 0.03           | 6.65E-01 | -0.05          | 4.43E-01                         | Opposite                         |
| RLN1   | Alternative promoter     | Repression of promoter 2     | -0.22          | 1.82E-05 | --             | --       | --             | --                               | Not assessed                     |
| AP2S1  | Alternative promoter     | Induction of promoter 2      | -0.04          | 5.51E-01 | --             | --       | --             | --                               | Not assessed                     |
| RLN2   | Alternative promoter     | Induction of promoter 1      | -0.16          | 5.68E-03 | --             | --       | --             | --                               | Not assessed                     |
| PIK3R1 | Alternative promoter     | Repression of promoter 2     | -0.02          | 7.92E-01 | 0.11           | 5.92E-02 | -0.14          | 3.27E-02                         | Opposite                         |
| MAPRE2 | Alternative promoter     | Switch to promoter 2         | -0.02          | 7.56E-01 | -0.02          | 6.82E-01 | 0.03           | 1.00E+00                         | Inconclusive                     |
| NDUFAF4| Alternative promoter     | Repression of promoter 2     | 0.08           | 1.89E-01 | --             | --       | --             | --                               | Not assessed                     |
| DCXR   | Alternative promoter     | Repression of promoter 2     | -0.30          | 6.32E-10 | --             | --       | --             | --                               | Not assessed                     |
| PEX10  | Alternative promoter     | Switch to promoter 2         | 0.10           | 9.95E-02 | --             | --       | --             | --                               | Not assessed                     |
| SNAPC2 | Alternative promoter     | Switch to promoter 2         | 0.16           | 4.77E-03 | --             | --       | --             | --                               | Not assessed                     |
| ATP6V0D1| Alternative promoter     | Repression of promoter 2     | -0.11          | 5.43E-02 | --             | --       | --             | --                               | Not assessed                     |
| ARRD1C | Alternative promoter     | Induction of promoter 2      | 0.08           | 2.06E-01 | --             | --       | --             | --                               | Not assessed                     |
| DENND1A| Alternative promoter     | Repression of promoter 2     | -0.01          | 8.49E-01 | --             | --       | --             | --                               | Not assessed                     |
| KLH36  | Alternative promoter     | Induction of promoter 2      | -0.10          | 1.04E-01 | --             | --       | --             | --                               | Not assessed                     |
| RAB3IL1| Alternative promoter     | Repression of promoter 2     | 0.08           | 1.71E-01 | 0.33           | 4.58E-12 | 0.00           | 9.75E-01                         | Opposite                         |
| ACER3  | Alternative promoter     | Repression of promoter 2     | 0.16           | 4.77E-03 | --             | --       | --             | --                               | Not assessed                     |
| OSBPL1A| Alternative promoter     | Induction of promoter 2      | 0.04           | 5.38E-01 | 0.13           | 1.59E-02 | -0.07          | 2.88E-01                         | Consistent                       |
| TRIM16 | Alternative promoter     | Induction of promoter 2      | 0.06           | 3.95E-01 | --             | --       | --             | --                               | Not assessed                     |
| VSIG10L| Alternative promoter     | Induction of promoter 1      | -0.12          | 5.43E-02 | --             | --       | --             | --                               | Not assessed                     |
| SEPT5  | Alternative promoter     | Repression of promoter 2     | 0.11           | 7.96E-02 | 0.07           | 2.54E-01 | -0.01          | 8.89E-01                         | Inconclusive                     |
| HMGCR  | Alternative promoter     | Repression of promoter 1     | 0.00           | 9.91E-01 | -0.04          | 5.77E-01 | 0.04           | 6.25E-01                         | Inconclusive                     |
| RDH13  | Alternative promoter     | Induction of promoter 1      | -0.03          | 7.33E-01 | 0.10           | 7.19E-02 | -0.12          | 9.32E-02                         | Inconclusive                     |
| GPRIN2 | Alternative promoter     | Repression of promoter 2     | --             | --       | 0.03           | 6.48E-01 | --             | --                               | Not assessed                     |
| CLK3   | Alternative promoter     | Repression of promoter 1     | -0.15          | 6.05E-03 | 0.02           | 7.76E-01 | 0.02           | 8.63E-01                         | Consistent                       |
| RNH1   | Alternative promoter     | Induction of promoter 1      | -0.02          | 7.92E-01 | 0.10           | 6.12E-02 | -0.08          | 2.28E-01                         | Inconclusive                     |
| ZFAND6 | Alternative promoter     | Repression of promoter 2     | 0.03           | 6.50E-01 | 0.04           | 5.78E-01 | -0.04          | 6.05E-01                         | Inconclusive                     |
| CDIP1  | Alternative promoter     | Repression of promoter 2     | 0.10           | 1.04E-01 | 0.02           | 7.82E-01 | 0.06           | 3.78E-01                         | Inconclusive                     |
| YIF1B  | Alternative promoter     | Switch to promoter 2         | -0.01          | 8.87E-01 | -0.10          | 6.71E-02 | 0.06           | 3.97E-01                         | Inconclusive                     |
| LIMK2  | Alternative promoter     | Switch to promoter 2         | 0.00           | 9.67E-01 | -0.05          | 4.72E-01 | 0.00           | 9.75E-01                         | Inconclusive                     |
| TSC22D3| Alternative promoter     | Repression of promoter 1     | -0.13          | 3.44E-02 | -0.07          | 2.54E-01 | -0.03          | 6.59E-01                         | Consistent                       |
| ALDH1A3| Alternative promoter     | Repression of promoter 1     | -0.18          | 7.69E-04 | --             | --       | --             | --                               | Not assessed                     |
| TRABD  | Alternative promoter     | Switch to promoter 2         | 0.06           | 3.95E-01 | -0.03          | 6.48E-01 | 0.03           | 7.83E-01                         | Inconclusive                     |
| LIMCH1 | Alternative promoter     | Repression of promoter 2     | 0.02           | 7.85E-01 | --             | --       | --             | --                               | Not assessed                     |
| GMFB   | Alternative promoter     | Induction of promoter 2      | 0.07           | 2.57E-01 | --             | --       | --             | --                               | Not assessed                     |
| MLST8  | Alternative promoter     | Switch to promoter 1         | 0.10           | 8.19E-02 | 0.15           | 6.14E-03 | 0.02           | 7.83E-01                         | Inconclusive                     |
| TLE3   | Alternative promoter     | Induction of promoter 2      | 0.03           | 6.38E-01 | -0.11          | 3.84E-02 | 0.04           | 5.65E-01                         | Opposite                         |
| UBA1   | Alternative promoter     | Repression of promoter 1     | 0.12           | 5.43E-02 | 0.00           | 9.72E-01 | 0.06           | 3.99E-01                         | Inconclusive                     |
| Gene          | Event type         | Change with androgens (LNCap) | Isoform 1 | Isoform 2 | PSI | Consistency of change with stage |
|--------------|--------------------|-------------------------------|-----------|-----------|-----|----------------------------------|
| TNRC6B       | Alternative promoter| Repression of promoter 2      | -0.04     | 6.31E-01  | -0.03 | 6.48E-01 | 0.02 | 7.83E-01 | Inconclusive |
| FDXT1        | Alternative promoter| Repression of promoter 2      | -0.05     | 4.82E-01  | 0.04  | 5.46E-01 | -0.08 | 2.28E-01 | Inconclusive |
| GREB1        | Alternative promoter| Induction of promoter 2       | -0.11     | 7.48E-02  | -0.18 | 7.01E-04 | 0.01  | 8.96E-01 | Inconclusive |
| NCAPD3       | Alternative promoter| Induction of promoter 2       | -0.23     | 1.82E-05  | --    | --     | --    | --     | Not assessed |
| SLC36A4      | Alternative promoter| Induction of promoter 2       | 0.07      | 2.59E-01  | --    | --     | --    | --     | Not assessed |
| KLC2         | Alternative promoter| Repression of promoter 1      | -0.03     | 6.33E-01  | 0.13  | 1.81E-02 | -0.08 | 2.78E-01 | Inconclusive |
| RAP1GAP      | Alternative promoter| Repression of promoter 1      | 0.02      | 7.85E-01  | --    | --     | --    | --     | Not assessed |
| TMEM79       | Alternative promoter| Repression of promoter 1      | -0.08     | 1.71E-01  | 0.16  | 1.97E-03 | -0.10 | 1.20E-01 | Inconclusive |
| NR4A1        | Alternative promoter| Induction of promoter 2       | 0.01      | 8.49E-01  | -0.06 | 3.69E-01 | 0.08  | 2.62E-01 | Inconclusive |
| ZNF32        | Alternative promoter| Repression of promoter 2      | -0.15     | 6.70E-03  | 0.02  | 7.34E-01 | -0.08 | 2.33E-01 | Inconclusive |
| C1QTNF3      | Alternative promoter| Induction of promoter 1       | 0.03      | 6.74E-01  | --    | --     | --    | --     | Not assessed |
| UBE2D3       | Alternative promoter| Switch to promoter 2          | 0.20      | 2.96E-04  | 0.07  | 2.17E-01 | -0.02 | 7.83E-01 | Inconclusive |
| KRT8         | Alternative promoter| Repression of promoter 1      | -0.04     | 6.05E-01  | -0.24 | 2.72E-06 | 0.04  | 6.05E-01 | Inconclusive |
| ELOVL1       | Alternative promoter| Induction of promoter 2       | 0.13      | 2.87E-02  | --    | --     | --    | --     | Not assessed |
| RAN1         | Alternative promoter| Induction of promoter 2       | 0.09      | 1.26E-01  | -0.01 | 8.69E-01 | 0.10  | 1.20E-01 | Inconclusive |
| SORBS3       | Alternative promoter| Induction of promoter 2       | 0.11      | 7.96E-02  | --    | --     | --    | --     | Not assessed |
| MAT2A        | Alternative 3' end  | Repression of isoform 2       | 0.01      | 9.35E-01  | 0.18  | 7.83E-04 | -0.21 | 8.42E-05 | Opposite    |
| C1QTNF3      | Alternative 3' end  | Induction of isoform 1        | 0.05      | 3.95E-01  | 0.05  | 4.47E-01 | -0.04 | 6.05E-01 | Inconclusive |
| TMEM125      | Alternative 3' end  | Induction of isoform 1        | --        | --        | 0.16  | 2.80E-03 | --    | --     | Not assessed |
| CBWD2        | Alternative 3' end  | Induction of isoform 2        | 0.08      | 1.74E-01  | --    | --     | --    | --     | Not assessed |
| NDUVF3       | Alternative exon    | Switch to isoform 2 (exon excluded) | 0.11     | 7.48E-02  | -0.05 | 4.72E-01 | 0.11  | 1.00E-01 | Inconclusive |
| ZNF678       | Alternative exon    | Switch to isoform 2 (exon excluded) | -0.02    | 7.43E-01  | --    | --     | --    | --     | Not assessed |
| ZNF121       | Alternative exon    | Switch to isoform 2 (exon excluded) | -0.08    | 1.80E-01  | 0.03  | 6.48E-01 | -0.09 | 1.82E-01 | Inconclusive |
| SPATC1L      | Alternative exon    | Induction of isoform 2 (exon included) | -0.10    | 9.95E-02  | --    | --     | --    | --     | Not assessed |
| MOCOS        | Alternative exon    | Switch to isoform 2 (exon included) | 0.03     | 6.33E-01  | --    | --     | --    | --     | Not assessed |
| RBM45        | Alternative exon    | Switch to isoform 2 (exon included) | 0.08     | 1.71E-01  | --    | --     | --    | --     | Not assessed |
| MIPEP        | Alternative exon    | Repression of isoform 2 (exon excluded) | -0.16    | 4.48E-03  | --    | --     | --    | --     | Not assessed |
| BBS4         | Alternative exon    | Induction of isoform 2 (exon included) | -0.06    | 3.85E-01  | --    | --     | --    | --     | Not assessed |
| FAM195A      | Alternative exon    | Switch to isoform 1 (exon included) | 0.06     | 3.37E-01  | 0.10  | 6.85E-02 | -0.10 | 1.20E-01 | Inconclusive |
| LINCO1133    | Alternative exon    | Induction of isoform 1 (exon included) | 0.06     | 5.68E-01  | -0.04 | 5.46E-01 | 0.06  | 3.97E-01 | Inconclusive |
| SS18         | Alternative exon    | Switch to isoform 2 (exon included) | 0.15     | 6.05E-03  | 0.11  | 3.84E-02 | 0.11  | 1.00E-01 | Inconclusive |
| RHOC         | Alternative exon    | Switch to isoform 2 (exon included) | -0.03    | 6.64E-01  | -0.09 | 1.23E-01 | 0.07  | 3.35E-01 | Inconclusive |
| ZNF226       | Retained intron     | Switch to isoform 1 (intron included) | -0.03    | 6.64E-01  | -0.09 | 1.23E-01 | 0.07  | 3.35E-01 | Inconclusive |
Data availability
The RNASeq data from LNCaP cells has been published previously [1]. The RNAseq custom tracks are available in Supplementary File 1. To view these files please load them onto the UCSC website using the ‘My data’ tab and ‘custom tracks’. Then ‘Paste URLs or data’. The data is aligned to Feb 2009 (GRCh37/hg19).

Prostate adenocarcinoma cohort RNA-Seq data was downloaded from the Broad Institute TCGA Genome Analysis Center: Firehose 16/01/28 run [2].

Dataset 1: Real-time PCR raw Ct values 10.5256/f1000research.15604.d212873[3]

Dataset 2: Raw unedited western blot images 10.5256/f1000research.15604.d212874[4]

Competing interests
No competing interests were disclosed.

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Supplementary material
Supplementary Table 1: Details of primer sequences used. Click here to access the data.

Supplementary File 1: RNA-Seq reads custom tracks for visualisation on UCSC genome browser. Click here to access the data.

Supplementary Figure 1: PCR validation of 17 androgen regulated alternative events. Click here to access the data.

Supplementary Figure 2: Differential alternative mRNA isoform expression in the TGCA PRAD cohort. Normal vs. tumour (unpaired samples) analysis. Violin-boxplots of expression in transcripts per million mapped reads (TPM) of Isoforms 1 (left panel) and 2 (central panel), and of their expression ratio in PSI (right panel) in normal and tumour samples. The mean log2 fold-change (logFC) in expression between tumour and normal samples and the associated FDR-adjusted p-value for the moderated t-statistic of differential expression are shown for both isoforms (left and central panels). The mean difference in PSI (deltaPSI) between tumour and normal samples and the associated FDR-adjusted p-value for the Mann-Whitney U test of differential splicing are shown (right panel). Click here to access the data.

Supplementary Figure 3: Differential alternative mRNA isoform expression in the TGCA PRAD cohort across different Gleason grades. Violin-boxplots of expression in transcripts per million mapped reads (TPM) of Isoforms 1 (left panel) and 2 (central panel), and of their expression ratio (right panel) by Gleason grade. Their respective Spearman’s correlation coefficient (Rho) with grade and associated FDR-adjusted p-value are shown. Click here to access the data.

Supplementary Figure 4: Differential alternative mRNA isoform expression in the TGCA PRAD cohort across different tumour stages. Violin-boxplots of expression in transcripts per million mapped reads (TPM) of Isoforms 1 (left panel) and 2 (central panel), and of their expression ratio (right panel) by tumour stage. Their respective Spearman’s correlation coefficient (Rho) with stage and associated FDR-adjusted p-value are shown. Click here to access the data.

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Current Referee Status: ✔ ✔

Version 1

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This paper by Munkley and colleagues identifies in a comprehensive manner novel alternative mRNA isoforms regulated by androgens. Interestingly most isoforms result from a choice between alternative promoters, suggesting that regulation takes place mostly at the transcriptional level, but they identified also a few alternative cassette exons and 3' ends. They show experimental validation for 17 isoforms. Beside increasing the number of identified genes in the context of androgen-treated prostate cancer LNCaP cells, the authors analysed the expression of those new isoforms in a large cohort of prostate tumours. They found the expression of some of the mRNA isoforms is positively correlated in the androgen-treated cell and in cancer versus normal samples, and find further correlation with the tumour grade and stage for 3 alternative isoforms.

Overall this is an interesting work that clearly deserves to be published, as it reveals new potentially interesting target genes for prostate cancer. I have only a couple of comments/questions that may help to improve the strength of the manuscript.

Did the authors try to experimentally validate the regulation of alternative isoforms for the 3 most interesting genes, i.e. OSBPL1A, CLK3 and TSC22D3, which is correlated to tumour stage? As these new isoforms are predicted to alter the protein sequence, is it possible to discuss or predict what could be the impact of these modifications for these proteins, with regards to what is known about their function and/or in the context of prostate cancer?

Looking at the RNA-seq profiles for the validated examples, it seems to me that in some cases, especially for RLN1 and RLN2, regulation of promoter choice correspond also to changes in the 3 end of the transcript (the peak seems to be shifted to the 3' end). Such examples may have escaped the in silico prediction, but can you make any comment on this?

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes
Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Transcription and alternative splicing, transcriptomics

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Prostate cancer (PCa) is still a significant health problem in UK and across the world. Though a small minority of patients progress to aggressive forms, the absolute number is quite significant due to the high incidence of PCa among men. Therefore, investigation of molecular mechanisms of PCa progression is very important and will hopefully unravel novel therapeutic targets.

Alternative splicing (AS) has been shown to occur in over 94% of genes in humans. It is therefore a crucial level of gene regulation and not surprisingly involved in virtually every physiological and pathological process. AS de-regulation has been implicated in many diseases, including cancer and in particular PCa, and interestingly, many times it has been shown to drive cancer pathology independently of transcription.

Since androgens are main players in PCa, the idea of analysing global changes in AS in response to androgens is very welcome to the field. The authors found 10 times more AS isoforms regulated by androgens than previously reported in data from cell culture, most of them occurring through alternative promoter mechanism. They have confirmed and validated part of these changes. They have also analysed the isoforms changes between adenocarcinoma and normal tissues as well as during progression through the Gleason stages of PCa.

This is a very well thought and executed study, with may informative results. I have a suggestion for the discussion part:

- one issue in global analysis of splice isoforms is which ones are causal (ie maintain and aggravate the phenotype) and which ones are just associated with the pathological progression; while a full...
answer to this would need experimental evidence on each individual splicing event, could the authors discuss 1-2 examples, if possible, where the changes at protein level (either sequence or expression level of a particular isoform) would hypothetically have a causal role

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: alternative splicing; prostate cancer; diabetes (renal complications)

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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