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# c-Myc Antagonises the Transcriptional Activity of the Androgen Receptor in Prostate Cancer Affecting Key Gene Networks

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ABSTRACT

Prostate cancer (PCa) is the most common non-cutaneous cancer in men. The androgen receptor (AR), a ligand-activated transcription factor, constitutes the main drug target for advanced cases of the disease. However, a variety of other transcription factors and signaling networks have been shown to be altered in patients and to influence AR activity. Amongst these, the oncogenic transcription factor c-Myc has been studied extensively in multiple malignancies and elevated protein levels of c-Myc are commonly observed in PCa. Its impact on AR activity, however, remains elusive. In this study, we assessed the impact of c-Myc overexpression on AR activity and transcriptional output in a PCa cell line model and validated the antagonistic effect of c-MYC on AR-targets in patient samples. We found that c-Myc overexpression partially reprogrammed AR chromatin occupancy and was associated with altered histone marks distribution, most notably H3K4me1 and H3K27me3. We found c-Myc and the AR co-occupy a substantial number of binding sites and these exhibited enhancer-like characteristics. Interestingly, c-Myc overexpression antagonised clinically relevant AR target genes. Therefore, as an example, we validated the antagonistic relationship between c-Myc and two AR target genes, KLK3 (alias PSA, prostate specific antigen), and Glycine N-Methyltransferase (GNMT), in patient samples. Our findings provide unbiased evidence that MYC overexpression deregulates the AR transcriptional program, which is thought to be a driving force in PCa.



## 1. Introduction

Prostate cancer (PCa) is the most common non-cutaneous cancer in men and the second most common cause of cancer-related deaths. Genomic alterations and changes in transcriptional regulation are keys to PCa initiation and progression and a crucial factor is dysregulated androgen receptor (AR) activity (Barfeld et al., 2014a). The AR is a ligand-activated transcription factor that controls key cellular processes including anabolic metabolism and cell cycle control (Barfeld et al., 2014b; Massie et al., 2011). Despite AR-targeted therapies, most advanced cases of PCa still maintain an active AR signaling network, presumably due to a range of activating mutations, gene amplifications and splicing events (Hu et al., 2012; Robinson et al., 2015; Taylor et al., 2010). Notably, genomic alterations affecting the AR directly appear to be limited to late stages of PCa (Taylor et al., 2010).

On the other hand, various other oncogenic signaling pathways and transcription factors are commonly mutated or amplified early in PCa. These include a) hyperactivation of the phosphoinositide 3-kinase (PI3K) pathway, b) translocations and fusions of a range of ETS transcription factors, such as ERG or ETV1, which place these factors under control of the AR, and c) amplification or overexpression of the oncogenic transcription factor c-Myc (MYC) (Robinson et al., 2015; Taylor et al., 2010).

The chromosome region containing the *MYC* gene (8q24) is commonly amplified in PCa, and several reports have confirmed elevated levels of *MYC* mRNA and protein in PCa patients (Gurel et al., 2008; Jenkins et al., 1997). Mechanistically, the work on MYC in PCa confirms its contribution to ribosome biogenesis and metabolism (Barfeld et al.,

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2015; Koh et al., 2011a). Whilst other transcription factors, such as ERG or ETV1, have been shown to antagonize and amplify AR-mediated transcriptional activity, respectively (Baena et al., 2013; Yu et al., 2010), the relationship between the AR and MYC in PCa is yet to be explored in detail. Therefore, in this study we mapped the genome-wide chromatin binding sites for MYC and AR in PCa cells and evaluated the effect of MYC overexpression on AR chromatin occupancy and transcriptional output. Developing a clearer understanding of the interplay between transcription factors in PCa is important in defining the correct context for biomarkers and therapeutic targets.

## 2. Materials and Methods

### 2.1. Cell Culture and Manipulation

The LNCaP-MYC (Ramos-Montoya et al., 2014) and the corresponding empty vector (EV) line were cultured at 37 °C and 5% $CO_2$ in RPMI1640 (Gibco, 21875), containing 10% fetal bovine serum (FBS) (Gibco, 10500) and 2 μg/ml puromycin and 200 μg/ml G418 (Gibco, 10131019) for plasmid maintenance. For hormone starvation, cells were washed once with PBS (Gibco, 10010) and cultured in phenol red-free RMPI1640 (Gibco, 11835063), containing 10% charcoal-stripped FBS (Gibco, 12676029) for 72 h before starting the experiment. MYC overexpression was induced using 2 μg/ml doxycycline. Parental LNCaP cells were cultured under the same conditions minus the antibiotics. VCaP cells were cultured in DMEM (Gibco), containing 10% FBS under the same conditions.

For viability assays, the amount of viable cells was determined using Cell Aequous solution MTS reagent (Promega, G3581) following the manufacturer's recommendations.

Sarcosine levels were determined using a Sarcosine Assay Kit (Abcam, ab65338) following the manufacturer's recommendations.

Reverse siRNA transfection was performed using the Lipofectamine RNAiMAX transfection reagent (Life technologies, 13778150) and OptiMEM transfection medium (Life technologies, 31985-070). The following siRNAs were used: ON-TARGETplus Non-Targeting Pool (Thermo Scientific, D-001810-10) and ON-TARGETplus Human MYC SMARTpool (Thermo Scientific, L-003282-02).

### 2.2. ChIP-exo/-seq and Analysis

ChIP-exo and ChIP-seq were performed as previously described (Massie et al., 2011; Serandour et al., 2013). Antibodies used were AR (scbt, sc-816x), MYC (R&D, AF3696), H3K4me1 (Diagenode, pAb-194-050), H3K4me3 (Diagenode, C154100003), H3K27ac (Diagenode, pAb-196-050), H3K27me3 (Diagenode, pAb-195-050) and IgG control (scbt, sc-2027). Briefly, cultured LNCaP MYC cells were crosslinked, quenched, lysed and the chromatin sheared to an average size of approximately 200–300 bp. Following overnight incubation with specific antibodies or an IgG control, several on-bead enzymatic reactions including two exonuclease digestions were performed prior to overnight crosslink reversal and elution. DNA was cleaned-up and subjected to final enzymatic reactions. The resulting Illumina-compatible libraries were single-end sequenced on Illumina HiSeq 2000 instruments (Illumina). For ChIP-seq experiments, Illumina libraries were prepared using the TruSeq kit and single-end sequenced on Illumina HiSeq 2000 instruments (Illumina), as previously described (Massie et al., 2011).

The raw reads were aligned using novoalign (http://www.novocraft.com) or bowtie (for histone ChIPs) with default parameters on the human genome version 19 (hg19). Filter to SAM quality 20 was applied and only a maximum of 5 duplicated reads were kept. The peak detection (i.e. binding site detection) was performed using MACS with default parameters using inputs samples as controls (Zhang et al., 2008). Only reproducible peaks (i.e. those that occurred in both replicates) were considered in downstream analyses.

To assess the presence of motifs of other transcription factors in the ChIP-exo or ChIP-seq dataset, we looked for overrepresented TF motifs using "findMotifsGenome.pl", and peaks distribution analysis with annotatePeaks.pl, both parts of the HOMER package. Read distribution analysis was performed using an in-house script (Urbanucci et al., 2012), which generated a matrix of "normalized differences between coverage integrals in treated versus control samples aligned reads" using a 2000 bp window around peaks. Normalization was computed as 10 M/dataset size. Other downstream analyses were performed using the Galaxy platform and the CEAS package.

To obtain the MYC binding profile which we called "MYC ENCODE compendium", we created a custom bed file containing MYC peaks present in any of the following cell lines used by the ENCODE (Consortium, 2004) (https://genome.ucsc.edu/): HeLa, H1-ESC, K562, HepG2 and HUVEC.

### 2.3. Quantitative Real Time PCR (qRT-PCR) and ChIP qPCR

Total RNA was isolated using the Qiagen RNeasy kit (Qiagen, 74106) following the manufacturer's recommendations. RNA concentration and purity was measured using a NanoDrop instrument (Thermo Scientific). 500 ng to 1 μg total RNA were reverse transcribed using the SuperScript VILO kit (Applied Biosystems, 11754) following the manufacturer's recommendations. qRT-PCR was performed using SYBR green master mix (Applied Biosystems, 4385612). Amplification was performed in duplicate series using the ABI 7900HT FAST Sequence Detection System (Applied Biosystems) with the following cycling conditions, 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Transcript levels were normalized to vehicle controls and the expression levels of beta-actin using the 2^ddCt method. A complete list of primers can be found in Table S1.

### 2.4. Expression Arrays and Analysis

For microarray analysis, RNA integrity was confirmed using a 2100 Bioanalyzer (Agilent) and Total RNA Nano Chip (Agilent, 5067–1511). 500 ng RNA were reverse transcribed and Biotin-labeled using the TotalPrep-96 RNA Amplification kit (Illumina, 4393543) following the manufacturer's recommendations. Resuspended cRNA samples were hybridized onto Human HT-12 Expression BeadChips (Illumina, BD-103-0204). Missing probes were imputed using Illumina's GenomeStudio Gene Expression Module. The imputed probe datasets were analyzed using the freely available J-Express 2012 software (http://jexpress.bioinfo.no/site/). The raw data was quantile normalized and log2 transformed prior to analysis. Differential expression analysis was performed using the grouped triplicate experiments and Rank product analysis. Probes with a q-value of $<0.1$ were considered significantly up- or downregulated. For hierarchical clustering using complete linkage and Pearson correlation, differentially expressed probes were merged and high level mean and variance normalized. Heatmaps were drawn using Java treeview.

Gene Set Enrichment Analysis (GSEA) is a bioinformatic method that is used to assess whether sets of genes are significantly different. The method computes the similarity between a query gene-set compared to the gene-sets available in the GSEA database and derived from published studies. For GSEA, the javaGSEA Desktop Application (http://www.broadinstitute.org/gsea/index.jsp) was used with the following gene set collections: c2: curated gene sets. An additional dataset was included in the analysis for the purpose of this study: an AR signature derived from Asangani et al. (Asangani et al., 2014).

KEGG and GO pathway analyses were performed using the genecodis tool (http://genecodis.cnb.csic.es/) (Carmona-Saez et al., 2007).

To identify statistically significant biochemical recurrence courses, recursive partitioning was performed on a single gene expression profile using the 'party' package from CRAN (Han et al., 2012) with

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