O-GlcNAc Transferase Integrates Metabolic Pathways to Regulate the Stability of c-MYC in Human Prostate Cancer Cells

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Abstract

Metabolic disruptions that occur widely in cancers offer an attractive focus for generalized treatment strategies. The hexosamine biosynthetic pathway (HBP) senses metabolic status and produces an essential substrate for O-linked β-N-acetylglucosamine transferase (OGT), which glycosylates and thereby modulates the function of its target proteins. Here, we report that the HBP is activated in prostate cancer cells and that OGT is a central regulator of c-Myc stability in this setting. HBP genes were overexpressed in human prostate cancers and androgen regulated in cultured human cancer cell lines. Immunohistochemical analysis of human specimens (n = 1987) established that OGT is upregulated at the protein level and that its expression correlates with high Gleason score, pT and pN stages, and biochemical recurrence. RNA interference–mediated silencing or pharmacologic inhibition of OGT was sufficient to decrease prostate cancer cell growth. Microarray profiling showed that the principal effects of OGT inhibition in prostate cancer cells were related to cell-cycle progression and DNA replication. In particular, c-MYC was identified as a candidate upstream regulator of OGT target genes and OGT inhibition elicited a dose-dependent decrease in the levels of c-MYC protein but not c-MYC mRNA in cell lines. Supporting this relationship, expression of c-MYC and OGT was tightly correlated in human prostate cancer samples (n = 1306). Our findings identify HBP as a modulator of prostate cancer growth and c-MYC as a key target of OGT function in prostate cancer cells. Cancer Res 73(16); 5277–87. ©2013 AACR.

Introduction

Prostate cancer is the second most common male cancer in the world. The androgen receptor (AR) is a principal target in prostate cancer research because AR activity is maintained in castration-resistant disease, and both localized and advanced diseases are responsive to drugs that alter hormonal signaling (1). AR regulates anabolic metabolism and promotes aerobic glycolysis (2, 3). Experiments carried out in cell lines have led to the identification of large metabolic networks and the question remains whether there is an integration point of these networks that is clinically relevant.

The hexosamine biosynthetic pathway (HBP) requires glutamine, glucose, acetyl-Coenzyme-A, and nucleotide UTP to synthesize UDP-N-acetyl-D-glucosamine (UDP-GlcNAc; ref. 4). This pathway senses the availability of energy and couples metabolic flux to control cell proliferation (4–6). HBP provides substrate for posttranslational modification of plasma membrane and secretory proteins. In addition, UDP-GlcNAc is used by O-linked β-N-acetylgalactosamine (O-GlcNAc) transferase (OGT) that modifies target proteins in cytosol, mitochondria, and nucleus (7). Consequently, the HBP has emerged as a versatile regulator of signaling cascades influencing cell cycle (8), growth (5), metabolism (6, 9), and stress (7, 10). OGT has previously been reported to be overexpressed in a range of cancers, such as breast cancer (11) and lung and colon cancers (12). Knockdown of OGT reduces tumor growth in breast cancer mouse model (11) and invasion in colon cancer cell lines (12), suggesting that OGT activity contributes to the transformation phenotype. Some reports suggest that OGT serves similar role in prostate cancer cell lines, in part, through the O-linked glycosylation of FOXM1 (13), but no study has yet explored the regulation of the rate-limiting steps in the HBP pathway or assessed the effect of OGT inhibition in an unbiased way.

Materials and Methods

Prostate tissue specimens for mRNA analysis

Matched benign and malignant prostate tissues were derived from radical prostatectomy of 29 patients with prostate cancer, treated at Oslo Urological University Clinic (Oslo,
blocks each containing 129 to 522 tumor samples. For internal controls, each TMA block also contained different various control tissues, including normal prostate tissue.

**Immunohistochemistry.** Following immunohistochemistry (IHC) optimization for OGT (mouse monoclonal; Novus Biologicals #NB300-524; dilution 1:1350), the TMA was immunostained. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at pH 9.0. Bound primary antibody was visualized using the DAKO EnVision Kit (DAKO).

Nuclear OGT staining (0, +1, +2, +3) was scored for each tissue spot. This scoring pattern has been used previously (17). Tissue samples without definite prostate cancer were excluded.

**Statistical analysis.** Statistical calculations were conducted with JMP statistical software (Version 8.0, SAS institute). Contingency tables were calculated with the $\chi^2$ test and Fisher exact test to analyze differences between groups. Survival curves were calculated by the Kaplan–Meier method and compared with the log-rank test.

**Technical issues.** As in all TMA studies, a fraction of the cases were noninformative due to complete lack of tissue samples ($n = 475$) or absence of unequivocal cancer tissue ($n = 799$).

**Cell lines and maintenance**

Cells were obtained from the American Tissue Culture Collection (ATCC) and maintained according to ATCC guidelines. Cell lines were authenticated by the provider and were used within 6 months receipt. LNCaP and PC-3 cells were grown in RPMI media supplemented with 10% FBS. VCaP cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS. RWPE-1 cells were maintained in Keratinocyte-SFM (KSFM) media supplemented with EGF and bovine pituitary extract, according to manufacturer’s instructions. Before R1881 stimulation, cells were maintained in phenol red-free media supplemented with 10% charcoal-stripped serum for 72 hours (LNCaP and VCaP), whereas RWPE-1 cells were maintained in KSF M media supplemented with 0.5% bovine serum albumin. The viability of cells was assessed with an MTS assay (Promega) according to manufacturer’s instructions.

**Preparation of cell lysates and Western blotting**

All the steps were conducted at 4°C, unless otherwise mentioned. Cells were washed once with PBS and harvested into cell lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 1% Triton X-100, 0.1% sodium-deoxycholate, 0.1% SDS, 140 mmol/L NaCl + Complete protease inhibitor mixture; Roche), rotated for 15 minutes and centrifuged 14,000 g 10 minutes. Supernatant was collected and protein concentration determined with bicinchoninic acid (BCA) assay. Of note, 10 to 25 μg of lysate was separated with SDS-PAGE, using 4% to 12% gradient gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed with antibodies against OGT (Santa Cruz), GFP-T1, ACTIN, β-tubulin, UAP1 (Sigma), MYC (Epitomic), and IL2 (Abcam). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies against cognate

**Tissue microarray and immunohistochemistry**

**Patient cohort.** Radical prostatectomy specimens were available from 3,261 patients, treated in the Department of Urology, University Medical Center Hamburg-Eppendorf (Hamburg, Germany) between 1992 and 2008 (14). Follow-up data were available for 3,057 patients, ranging from 0.1 to 228.7 months (mean 101.6 months). None of the patients received neoadjuvant endocrine therapy. Additional (salvage) therapy with 4

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species (Dako). The intensity of the signals from each antibody was quantified by Quantity One software (Bio-Rad).

**Immunoprecipitation and lectin pulldown**

All the steps were conducted at 4°C. Cells were washed once with PBS and solubilized in cell lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 1% Triton X-100, 0.1% Nadeoxycholate, 0.1% SDS, 140 mmol/L NaCl + Complete protease inhibitor mixture; Roche), rotated for 15 minutes and centrifuged 18,000 g for 5 minutes. Protein concentration was determined with BCA assay and 1,000 to 3,000 μg of protein was precleared with unspecific antibody (Santa Cruz) and protein A-goated magnetic beads (immunoprecipitation) or unbound agarose beads (Vector Labs) for 2 hours. Precleared extract was used for immunoprecipitation (R2 antibody, Abcam), or lectin pulldown (Wheat Germ Agglutinin, WGA, VectorLabs) overnight. Protein G-goated magnetic beads were added to the immunoprecipitation reaction, incubated for 2 hours and washed with immunoprecipitation wash buffer (0.5% NP-40, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0). Lectin pulldown was washed 3 times with lectin wash buffer (0.1% Tween, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0).

**RT-PCR and expression profiling of the cell lines**

RNA was collected by illustraMiniSpin (GE Healthcare) according to manufacturer's instructions. One microgram of RNA was used to produce cDNA (qScript cDNA Synthesis Kit, Quanta BioSciences). Subsequently, 0.3 μL was used for quantitative PCR (qPCR). Amplification was conducted as follows: 10 minutes 95°C followed by 40 cycles 30 seconds 60°C, 30 seconds extension, final extension 5 minutes in 72°C. Genes detected with SYBR Green are listed in the Supplementary Table S4 with corresponding primers. Housekeeping genes were detected with TaqMan assays (Applied Biosystems).

**Microarrays**

The purity and quantity of the extracted RNA were measured using the NanoDrop ND1000 spectrophotometer (Nanodrop Technologies), and the RNA integrity was evaluated using the Agilent 2100 Bioanalyzer with the RNA nano 6000 kit (Agilent Technologies Inc.). Synthesis of cDNA, cRNA amplification, and hybridization of cRNA onto the Illumina HumanHT-12 v4 Expression BeadChips (Illumina Inc.) was carried out as per manufacturer's instructions. Data extraction, quantile normalization, and initial quality control of the bead summary raw data were conducted using GenomeStudio v2011.1 from Illumina and the Gene Expression module v1.9.0. The data were annotated using the HumanHT-12_V4_0_R2_15002873_B.bgx annotation file from Illumina. Microarray data are deposited in Gene Expression Omnibus with an accession number GSE4624.

**Treatments**

Synthetic androgen, R1881 was solubilized in ethanol to a final concentration of 10 μmol/L. Inhibitor against human O-GlcNAc transferase, ST045849 (3-(2-adamantanyethyl)-2-[4-chlorophenyl]azamethylene]-4-oxo-1,3-thiazaperhyd roine-6-carboxylic acid), was purchased from TimTec and solubilized in dimethyl sulfoxide to final concentration of 20 mmol/L. OGT inhibitor has an IC50 value of 30 to 53 μmol/L, depending on the isoform of the enzyme (18) and it has been used to target OGT in 100 μmol/L (19). siRNAs targeting OGT (sOGT) were obtained from Lifetechologies (s16094 and s16095), and RNAiMax was used for the transfection.

**Results**

**Hexosamine biosynthetic pathway is upregulated during early stages of prostate cancer**

We have previously reported that the AR is a regulator of both metabolic and cell-cycle gene networks (2). In particular, we and others have found that genes associated with metabolic pathways are significantly overexpressed in prostate cancer (2, 3, 20). By combining publicly available chromatin immunoprecipitation (ChIP) data for 2 key transcription factors in prostate cancer (AR; ref. 2; ERG; ref. 21) and clinical gene expression data from 11 separate studies (20), we identified 195 genes, which have binding sites within their promoters and are overexpressed in the clinical setting (Supplementary Table S1). A pathway analysis on this genaset highlighted O-glycan biosynthesis and amino and sugar nucleotide metabolism as significantly deregulated processes (Supplementary Table S1). As a classifier, O-glycan biosynthesis encompasses complex sugar chains attached to plasma membrane proteins (22) and single O-GlcNAc modification occurring in other cell compartments (7). Importantly, single O-GlcNAc modification is catalyzed by only one enzyme in humans (OGT), which requires the activity of HBP and therefore functions as a metabolic integration point. The flux through HBP is controlled by the rate-limiting enzyme glutamine-fructose-6-phosphate transaminase 1 (GFTT1).

**Biomarker validation**

We sought to validate the transcript expression of the HBP genes in clinical samples. For this, we had access to 29 prostate cancer patient samples (matched tumor and normal tissue). We found that the enzymes catalyzing the rate-limiting step (GFTT1) and the enzyme catalyzing the final step in the HBP, UAPI (UDP-N-acetylglucosamine pyrophosphorylase 1) are upregulated in localized prostate cancer (P < 0.05; Fig. 1A). OGT is an integration point of HBP that is required to modify target proteins via O-linked glycosylation and acts as a critical regulator of protein stability and activity. We first confirmed that OGT is upregulated in localized prostate cancer in the mRNA level (Fig. 1A) and went on to assess the protein level expression of OGT in 1,987 clinically annotated prostate cancers by IHC (Fig. 1B and Table 1). Increased OGT expression correlated with increasing Gleason score and pT/pN stages (P < 0.0001), preoperative PSA (P < 0.01), and also with BCR (P < 0.0001; Table 1 and Fig. 1C).

**Regulation of the HBP pathway by the androgen receptor**

We further evaluated the linkage between AR and HBP gene expression in AR-expressing prostate cancer cell lines. LNCaP cells express a mutant variant of the AR (23), whereas VCaP harbors the TMPRSS2-ERG fusion gene and are also

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**Glycosylation Regulates the Stability of c-MYC**


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characterized by amplification and overexpression of wild-type AR (24). LNCaP and VCaP cells were deprived of androgens for 72 hours to minimize AR activity and then treated with a synthetic androgen (R1881) and mRNA was extracted after 18 hours. The expression of \( \text{GFPT1} \) and \( \text{UAP1} \) were increased by over 2-fold in both LNCaP and VCaP cells (Fig. 2A and B). The upregulation of \( \text{GFPT1} \) and \( \text{UAP1} \) was confirmed at the protein level in both cell lines (Fig. 2D and E).

To determine whether the hormone-dependent upregulation of the HBP enzymes is a feature found preferentially in cancer cell lines, we used RWPE-1 cells as a contrast control. This cell line was derived from normal prostate epithelia and expresses wild-type AR (25). RWPE-1 cells were cultured in the absence of growth factors for 48 hours to achieve a clear response to androgens, stimulated with R1881 and mRNA and protein lysates were collected. We observed no changes in the expression of genes associated with the HBP at the transcript (Fig. 2C) or protein levels (Fig. 2F).

**Targeting OGT with a small-molecule inhibitor or siRNA decreases cell viability**

Having established the cancer-specific upregulation of HBP enzymes, we wanted to inhibit this pathway and hypothesized that the most prominent target is OGT, which is positioned to integrate HBP activity to regulate target proteins and can be targeted with small-molecule inhibitors (18). We treated LNCaP and VCaP cells with a concentration gradient of an OGT inhibitor (ST045849) for 48 hours and assessed the viability using an MTS assay. OGT inhibitor caused dose-dependent decrease in the viability, and the highest concentration (corresponding to IC\(_{50}\); ref. 18) caused approximately 50% decrease in the viability in both cell lines (Fig. 3A and B). In addition, targeting OGT with siRNA reduced the growth rate of AR-positive prostate cancer cells (Supplementary Fig. S1A).

**Figure 1.** HBP is upregulated in human prostate cancer. A, mRNA samples were collected from prostate biopsies. In each case, both normal and cancerous tissue was collected. The expression of the genes of interest was determined with qPCR and normalized to 5 housekeeping genes (\( \text{GAPDH} \), \( \text{TBP} \), \( \text{ALAS1} \), \( \beta\)-actin, and 18S) and then to normal tissue. The significance of the expression was evaluated by paired samples t test, \( * \), \( P < 0.05; \) \( ** \), \( P < 0.01 \). B, evaluation of OGT staining in localized prostate cancer. OGT staining was classified into 4 groups (staining intensity 0 to +3), which were then used in the analysis. Examples of staining from each group are shown. \( ^{*} \), positive staining is obtained from the basal and stromal cells. C, Kaplan–Meier survival curve plotting BCR against postoperative time. The probability of biochemical relapse–free survival decreased in a dose-dependent manner with increasing OGT expression (\( P < 0.0001 \), across all intensities).
viability after 48 hours of treatment. LNCaP cells were treated with OGT inhibitor or siOGT for 12 hours and 24 hours to capture the processes affected shortly after the treatment. OGT inhibitor caused a 2-fold increase in the levels of OGT mRNA at 24 hours, whereas siOGT reduced the levels of OGT mRNA by 80% (Fig 3C). Biologic triplicate samples were analyzed by

Table 1. Clinical parameters of OGT staining

<table>
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<th>Parameter</th>
<th>n</th>
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<th>Int 1</th>
<th>Int 2/3</th>
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<td>PSA &lt;4</td>
<td>305</td>
<td>62 (20.3)</td>
<td>210 (68.9)</td>
<td>33 (10.8)</td>
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<td></td>
<td>4–10</td>
<td>1,047</td>
<td>232 (22.2)</td>
<td>710 (67.8)</td>
<td>105 (10)</td>
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<td></td>
<td>10–20</td>
<td>439</td>
<td>96 (21.9)</td>
<td>288 (65.6)</td>
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<td></td>
<td>&gt;20</td>
<td>155</td>
<td>30 (19.4)</td>
<td>91 (58.7)</td>
<td>34 (21.9)</td>
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<td>1,291</td>
<td>315 (24.4)</td>
<td>868 (67.2)</td>
<td>108 (8.4)</td>
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<tr>
<td>pT3a</td>
<td>401</td>
<td>66 (16.5)</td>
<td>270 (67.3)</td>
<td>65 (16.2)</td>
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<tr>
<td>pT3b</td>
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<td>41 (14.8)</td>
<td>176 (63.5)</td>
<td>60 (21.7)</td>
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<tr>
<td>pN Stage</td>
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<td>699 (69.1)</td>
<td>73 (7.2)</td>
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<td>567 (63.9)</td>
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<td>856</td>
<td>235 (27.5)</td>
<td>563 (65.8)</td>
<td>58 (6.8)</td>
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<td>3 + 4</td>
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<tr>
<td>4 + 3</td>
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<td>149 (70)</td>
<td>46 (21.6)</td>
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<tr>
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<td>2 (5.9)</td>
<td>15 (44.1)</td>
<td>17 (50)</td>
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<td>1,033 (66.8)</td>
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<td>&lt;0.0001</td>
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<td>281 (66.6)</td>
<td>75 (17.8)</td>
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NOTE: Samples were analyzed on the basis of the staining intensities (Int) shown in Fig. 1B. The number of patients falling into each group is reported and the percentage is shown in brackets. Reported P values represent those calculated across all classes.

Figure 2. AR regulates the expression of HBP enzymes in prostate cancer cell lines. Cell lines were deprived of androgens for 72 hours (LNCaP and VCaP) or 48 hours (RWPE-1) and stimulated with synthetic androgen (10 nmol/L R1881; ref. 47). A–C, mRNA levels after 18 hours of R1881 stimulation. Expression levels were normalized first to TBP and then to vehicle-treated condition. Data for LNCaP and VCaP cells are obtained from 2 biologic replicates and for RWPE-1 cells from 2 technical replicates. D–F, protein lysates were harvested at the indicated time points after R1881 stimulation and blotted for the proteins of interest. The intensity of Western blotting signals was determined with densitometry, normalized to β-tubulin, and the amount at 0 hours was set to one.
Figure 3. OGT regulates genes associated with cell-cycle and DNA replication. LNCaP (A) and VCaP (B) cells were treated with a concentration gradient of OGT inhibitor for 3 days and samples were analyzed with an MTS assay. The viability of vehicle-treated sample was set to 100% and other conditions were normalized to this. The values shown are from 4 technical replicates and SEM is shown.

C, LNCaP cells were treated either with siOGT or with a low dose of OGT inhibitor causing 20% decrease in the viability after 48 hours of treatment. RNA was harvested in biologic triplicates at each time point and the expression levels of genes of interest were determined with qPCR, normalized to actin and then to either vehicle-treated condition (OGT inhibitor) or scrambled control (siOGT).

D, expression profile was assayed using Illumina HT12v4 Bead Arrays. Data were quantile normalized using Bead Studio, and differentially expressed genes were identified using the JExpress software package. The 5 most upregulated genes (DDIT3, AKR1C2, ARHGEF2, AKR1C4, and FAM129A) or downregulated genes (CDC2, c11Orf82, ATAD2, MCM10, and ASF1B) from cells treated with OGT inhibitor for 24 hours were selected for validation of the data with qPCR. Expression of MYC mRNA was not changed by OGT inhibitor. The values were first normalized to TBP and then to vehicle-treated condition. E, the expression levels of the 20 most downregulated genes common to both time points after treatment with OGT inhibitor were compared with a published expression array data set (32). Within this dataset, there are a total of 218 cases and we selected the prostatectomy samples that later on developed into metastatic disease and compared these with the cases without prostate cancer (PAN) based on the clinical data described in Supplementary Table S1 of Taylor and colleagues (32).
expression arrays and data were processed using J-Express (28) to produce lists of up- and downregulated genes from each treatment and time point (Supplementary Table S2). We first evaluated the expression profiles after treating cells with siOGT. Strikingly, MGEA5, a gene encoding for O-GlcNAcase, was the topmost downregulated gene (after OGT) at 24 hours (Supplementary Table S2 and Supplementary Fig. S1B). This is in good agreement with previous reports showing that inhibition of OGT expression with siRNA is rapidly compensated by commensurate loss of MGEA5 (29). MGEA5 catalyses the removal of O-GlcNAc from OGT substrates and consequently this compensation effect underscores the importance of this pathway but also challenges the interpretation of OGT knockdown data.

We therefore concentrated on the data generated from cells treated with the OGT inhibitor. First, we took the 5 most up- or downregulated genes from 24 hours time point for validation with qPCR. We observed 1.5- to 5-fold increase in the levels of DDT3, AKR1C2, ARHGEF2, AKR1C4, and FAM129A (Fig. 3D). Next, we analyzed the expression levels of the 5 most downregulated genes CD2, c11orf82, ATRAD2, MCM10, and ASF1B, all of which, except for c11orf82, were downregulated by more than 50% at a 24-hour time point. To understand the significance of the altered gene expression profile, we conducted a pathway enrichment analysis with DAVID (30, 31) gene ontology enrichment tool (Supplementary Table S2). This approach revealed a consistent downregulation of gene networks associated with cell-cycle progression and DNA processing, upon treatment with OGT inhibitor.

We wanted to understand the clinical relevance of OGT inhibitor-induced changes in the expression profile, and therefore evaluated the expression of the 20 most downregulated genes common to both time points in clinical samples using data published in Taylor and colleagues (32). Interestingly, genes downregulated by OGT inhibitor were overexpressed in the prostate cancer tissue of patients with metastatic prostate cancer (Fig. 3E).

**Inhibition of OGT destabilizes c-MYC in prostate cancer cells**

OGT is a known epigenetic regulator (6), and we wanted to assess whether OGT inhibitor-induced changes in the transcriptome are mediated by specific transcription factors. We therefore took the 200 most downregulated genes common to both time points and uploaded them into the Ingenuity Pathway Analysis tool (Ingenuity Systems Inc, www.ingenuity.com) to look for common upstream regulators (Supplementary Table S2). The E2F family of transcription factors was the most prominent group of transcription factors discovered in this analysis. We next compared the expression levels of the candidate transcription factors with the expression levels of OGT in a large prostate cancer gene expression array dataset (32) and found that c-MYC is tightly coexpressed with OGT (Supplementary Fig. S2A). We wanted to take a more stringent approach and took only the 20 most downregulated genes common to both time points and used an analysis based on first neighbor associations with other factors as reported in the REACTOME. By these criteria, c-MYC was the sole transcription factor linked to the network (Supplementary Fig. S2C).

Consequently, we assessed the effects of OGT inhibitor on the stability of c-MYC. We first confirmed that OGT inhibitor was taken up by the cells by assessing the total O-GlcNAc signal in cell lysates after treatment with drug using blotting and densitometry. We observed a dose-dependent decrease in the levels of O-GlcNAc in 3 cell lines (LNCaP, VcaP, and PC3; Fig. 4A). The highest dose caused a minimum of 34% decrease in O-GlcNAc levels. The level of inhibition is similar to previously reported responses using the same drug (19). Next, we assessed the effects of OGT inhibitor on c-MYC andfound that inhibition of OGT results in loss of c-MYC in both AR-positive (LNCaP and VcaP) and AR-negative (PC3) prostate cancer cell lines (Fig. 4A). Treatment of cells with the OGT inhibitor did not reduce c-MYC mRNA levels (Fig. 3D), suggesting that OGT regulates c-MYC stability through a posttranscriptional mechanism. OGT activity can also be reduced by targeting the enzyme with siRNA and the levels of c-MYC decreased significantly after knocking down OGT for 96 hours (Supplementary Fig. S3A), c-MYC is known to be modified by O-GlcNAc modification (33, 34) and we confirmed this in prostate cancer cells with lectin- and immunoprecipitation-based enrichments (Supplementary Fig. S3B and S3C).

The data from prostate cancer cell lines support a link between OGT activity and c-MYC. The importance of this oncogenic transcription factor has been shown in a range of cancers, and amplification of the MYC locus has also been associated with poor prognosis in patients with prostate cancer (35). We speculated that OGT overexpression might associate with c-MYC copy number variation in the clinical setting to increase signaling via c-MYC oncogene in the lethal prostate cancer. The copy number status of MYC has been determined for 1,306 patients (35) in the cohort used to assess OGT expression here, which enabled us to test the potential association between MYC and OGT in the clinical setting. Interestingly, we observed a statistically significant association (P = 0.0012) between the increase in MYC copy number and OGT intensity (Fig. 4B). For the further validation, we went on to assess the potential coexpression between OGT and MYC in the mRNA level. Interestingly, we found a positive correlation between OGT and MYC, but not between OGT and AR or OGT and ERG (Fig. 4C).

**Discussion**

In this study, we used bioinformatics to identify dysregulated gene networks during the early stages of prostate cancer development. This approach led to the identification of HBP as a pathway capable of discriminating between benign prostate tissue and cancerous tissue. We confirmed the upregulation of the rate-limiting and the final enzymes in HBP in patients diagnosed with localized prostate cancer (Fig. 1A). These results are in good agreement with recent studies reporting GFPT1 as a candidate oncogene in pancreatic cancer (36). We also found elevated mRNA levels of
OGT in prostate cancer tissue and confirmed this result by IHC (Fig. 1B and C and Table 1). OGT and O-linked glycosylation have been frequently associated with cancer development (11–13, 19, 26, 37).

AR has been acknowledged as an important driver of prostate cancer (1), and we observed that both the rate-limiting enzyme (GFPT1) and the last enzyme (UAP1) are regulated by AR (Fig. 2A–F), which enables prostate cancer cells to maintain inappropriately high levels of the HBP enzymes. AR has been shown to activate multiple pathways in prostate cancer cells (2, 3), and the importance of our result lies in the identification of a confined AR-driven gene module integrating several aspects of metabolism to regulate cell proliferation (Fig. 5).

Targeting OGT with short hairpin RNA in mouse xenograft models decreases the growth of tumors (11, 13, 26). We found that the protein level expression of OGT correlates with high Gleason score and pN/T status in prostate cancer tissue and also with biochemical recurrence (Table 1 and Fig. 1C). These results position OGT as a candidate drug target and expression arrays revealed that treatment of cells with OGT inhibitor decreases the expression of genes associated with DNA replication and cell-cycle progression (Supplementary Table S2). We identified c-MYC as a candidate upstream regulator of OGT inhibitor-induced changes in gene expression, and confirmed this association both in cell lines and in clinical setting (Fig. 4). The importance of c-MYC for prostate cancer has been previously documented (38–41). Taken together, these results suggest that OGT activity integrates metabolic flux to regulate the stability of c-MYC (Fig. 5). This can be viewed as a variation into the genetic "2-hit" hypothesis (42), and we propose that increased OGT expression synergizes with MYC copy number gain to promote prostate cancer progression.

c-MYC is under both transcriptional and posttranslational control, and protein is known to be modified by O-linked glycosylation (33, 34). Recent therapeutic strategies to inhibit c-MYC expression and activity in cancers have included targeting bromodomain-containing proteins with small-molecule inhibitors. One example is a drug called JQ1, developed as an
inhibitor of BRD4 (43). This drug reduces c-MYC expression at the transcript level and has a significant impact on tumorigenesis in lymphoma cell lines and mouse models (44, 45). In contrast, the OGT inhibitor we used here reduced the protein level of c-MYC without impacting on c-MYC transcript expression (Figs. 3D and 4A). The stabilization of c-MYC has been reported to occur via serine/threonine phosphatase PP2A (46), and our work adds glycosylation as an alternative mechanism to stabilize c-MYC in cancer cells (Fig. 5). Future work will focus on the development of new reagents able to discriminate between glycosylated and nonglycosylated pools of this important oncogene.

Our data suggest that OGT activity supports the metabolic reprogramming of tumor cells in the clinical setting by synergizing with MYC copy number gain to maximize c-MYC activity. Taking further, our results support targeting OGT, or indeed c-MYC, in the treatment of prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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