Glycosylation regulates the stability of c-MYC

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 the hexosamine transferase OGT, which glycosylates and thereby modulates the function of its target proteins. Here we report that the hexosamine biosynthetic pathway (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 (GS), pT and pN stages and biochemical recurrence. RNAi-mediated silencing or pharmacological 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 related to cell cycle progression and DNA replication. In particular, c-MYC was identified as a candidate upstream regulator of OGT target genes and that OGT inhibition elicited a dose-dependent inhibition in the levels of c-MYC protein but not c-MYC mRNA in cell lines. In supporting of 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.

Precis

Targeting a protein glycosylation pathway that is dysregulated by metabolic flux in
Glycosylation regulates the stability of c-MYC cancer cells blocks MYC and inhibits cancer cell proliferation, possibly offering a broad-based anticancer strategy.
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 since AR activity is maintained in castration resistant disease and both localised and advanced diseases are responsive to drugs that alter hormonal signalling(1). AR regulates anabolic metabolism and promotes aerobic glycolysis(2, 3). Experiments performed 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)(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 utilized 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 signalling 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 tumour 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
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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 prostate cancer patients, treated at Oslo Urological University Clinic and obtained from “The ProstateBiobank - a resource for urological research in Norway” (http://www.ous-research.no/home/tasken/Research%20interests/10769). Clinical parameters of this prostate cancer patient cohort are available in Supplementary Table 3. Samples were treated with RNALater at 4°C overnight according to the manufactures instructions (Qiagen, Hilden, Germany) and stored at -80°C. The pathology of the samples was verified by uropathologist. Written consent was obtained from all patients and the project was approved by The Regional Committee for Medical and Health Research Ethics (REK sør-øst 2010/2218). 1μg of total RNA was reverse transcribed using the High-Capacity RNA-to-cDNA Master Mix (Applied BioSystems) according to manufacturer’s instructions. Quantitative Real-time PCR was performed using 1ng of cDNA with Fast SYBR Green PCR MasterMix (Applied Biosystems) for the detection of GCNT1, GFPT1, OGT, UAP1 (primer sequences Suppl. Table 4). TaqMan Gene expression Assays (Applied Biosystems) were used to determine the mRNA expression of the AR, ERG, MYC, GADPH, TBP, ALAS1, β-ACTIN and 18S. An ABI HT7900 Sequence Detection System was employed according to manufacturer’s recommendations. Each cDNA sample was tested in triplicate and mean Ct values were calculated. The mRNA expression level was determined using the comparative CT method and normalization to the geometric mean of GAPDH, ALAS1, 18s, ACTB and TBP. The expression level of the gene of interest in the normal tissue was set to the value of 1,
and the values for the matched tumor sample were calculated accordingly. The difference in mRNA expression between matched normal and tumor sample were tested by a dependent sample t-test. All data were analysed using SPSS v.19 software (SPSS, Chicago, IL, USA).

**Tissue Microarray (TMA) and Immunohistochemistry**

**Patient cohort:** Radical prostatectomy specimens were available from 3,261 patients, treated in the Department of Urology, University Medical Center Hamburg-Eppendorf 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 neo-adjuvant endocrine therapy. Additional (salvage) therapy was initiated in case of a biochemical relapse (BCR). In all patients, prostate specific antigen (PSA) values were measured quarterly in the first year, followed by biannual measurements in the second and annual measurements after the third year following surgery. Recurrence was defined as a postoperative PSA of 0.2 ng/ml and rising thereafter. The first PSA value above or equal to 0.2 ng/ml was used to define the time of recurrence. Patients without evidence of tumor recurrence were censored at the last follow-up. All prostatectomy specimens were analyzed according to a standard procedure. All prostates were completely paraffin-embedded, including whole-mount sections as previously described(15). All haematoxylin and eosin (H&E) stained histological sections from all prostatectomy specimens were reviewed and one 0.6 mm thick tissue core was punched out from a representative cancer area and transferred onto a TMA format as described(16). The 3,261 cores were distributed among 7 TMA blocks each
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containing 129 to 522 tumor samples. For internal controls, each TMA block also contained different various control tissues, including normal prostate tissue.

**Immunohistochemistry:** Following 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 performed with JMP statistical software (Version 8.0, SAS institute). Contingency tables were calculated with the $\chi^2$-test and Fisher’s exact test to analyze differences between groups. Survival curves were calculated by the Kaplan-Meier method and compared with the Logrank test.

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

**Cells 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
Glycosylation regulates the stability of c-MYC in RPMI media supplemented with 10% fetal bovine serum (FBS). VCaP cells were cultured in DMEM supplemented with 10% FBS. RWPE-1 cells were maintained in Keratinocyte-SFM media supplemented with EGF and BPE, 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), while RWPE-1 cells were maintained in KSFM media supplemented with 0.5% BSA. The viability of cells was assessed with an MTS assay (Promega) according to manufacturer’s instructions.

**Preparation of cell lysates and western blot**

All the steps were performed at 4°C, unless otherwise mentioned. Cells were washed once with PBS and harvested into cell lysis buffer (10mM Tris-Cl pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% TritonX-100, 0.1% sodium-deoxycholate, 0.1% SDS, 140mM NaCl + Complete protease inhibitor mixture, Roche), rotated for 15 minutes and centrifuged 14 000g 10 minutes. Supernatant was collected and protein concentration determined with BCA assay. 10-25μG of lysate was separated with SDS polyacrylamide gel electrophoresis, using 4-12% gradient gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed with antibodies against OGT (Santa Cruz), GFPT1, ACTIN, β-tubulin, UAP1 (Sigma), MYC (Epitomic), RL2 (Abcam). Primary antibodies were detected with HRP-conjugated secondary antibodies against cognate species (Dako). The intensity of the signals from each antibody was quantified by Quantity One software (Bio-Rad).
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Immunoprecipitation (IP) and lectin pulldown

All the steps were performed at 4°C. Cells were washed once with PBS and solubilized in cell lysis buffer (10mM Tris-HCl pH8.0, 1mM EDTA, 0.5mM EGTA, 1% TritonX-100, 0.1% Nadeoxycholate, 0.1% SDS, 140mM NaCl + Complete protease inhibitor mixture, Roche), rotated for 15 minutes and centrifuged 18 000g for 5 minutes. Protein concentration was determined with BCA assay and 1000-3000μG of protein was pre-cleared with unspecific antibody (Santa Cruz) and protein A-goated magnetic beads (IP) or un-bound agarose beads (VectorLabs) for 2 hours. Pre-cleared extract was used for IP (RL2 antibody, Abcam), or lectin pulldown (Wheat Germ Agglutinin, WGA, VectorLabs) overnight. Protein G-goated magnetic beads were added to the IP reaction, incubated for two hours and washed with IP wash buffer (0.5% NP-40, 150mM NaCl, 20mM Tris-HCl, pH8.0). Lectin pulldown was washed three times with lectin wash buffer (0.1% Tween, 150mM NaCl, 10mM Tris-HCl, pH8.0).

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, 0.3μl was used for qPCR. Amplification was performed as follows: 10min 95°C followed by 40 cycles 30 seconds 60°C, 30 seconds extension, final extension 5min in 72°C. Genes detected with SYBRgreen are listed in the supplementary table 4 with corresponding primers. Housekeeping genes were detected with TaqMan assays (AppliedBiosystems).
Microarrays

The purity and quantity of the extracted RNA were measured using the NanoDrop ND1000 spectrophotometer (Nanodrop Technologies, Delaware, USA), and the RNA integrity was evaluated using the Agilent 2100 Bioanalyzer with the RNA nano 6000 kit (Agilent Technologies Inc., California, USA). Synthesis of cDNA, cRNA amplification, and hybridisation of cRNA onto the Illumina HumanHT-12 v.4 Expression BeadChips (Illumina Inc, California, USA) was carried out as per manufacturer's instructions. Data extraction, quantile normalization and initial quality control of the bead summary raw data were performed 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 is deposited in Gene Expression Omnibus (GEO) with an accession number GSE44624.

Treatments

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

Hexosamine biosynthetic pathway is up-regulated 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 two key transcription factors in prostate cancer (AR(2) and ERG(21)) and clinical gene expression data from eleven separate studies(20), we identified 195 genes which have binding sites within their promoters and are overexpressed in the clinical setting (Suppl. Table 1). A pathway analysis on this geneset highlighted O-glycan biosynthesis and amino and sugar nucleotide metabolism as significantly deregulated processes (Suppl. Table 1). 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 GFPT1 (glutamine-fructose-6-phosphate transaminase 1).

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 tumour and normal tissue). We found that the enzymes catalyzing the rate-limiting step (GFPT1)
Glycosylation regulates the stability of c-MYC and the enzyme catalyzing the final step in the HBP, \textit{UAPI} (UDP-N-acetylglucosamine pyrophosphorylase 1) are up-regulated 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 \textit{OGT} is up-regulated in localized prostate cancer in the mRNA level (Fig. 1A) and went on to assess the protein level expression of OGT in one thousand nine hundred and eighty seven clinically annotated prostate cancers by immunohistochemistry (IHC) (Fig. 1B and Table 1). Increased OGT expression correlated with increasing Gleason Score and pT/pN stages (p<0.0001), pre-operative PSA (p<0.01) and also with biochemical relapse (p<0.0001, Table 1 and Fig. 1C).

\textbf{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) while VCaP cells harbour the \textit{TMPRSS2-ERG} fusion gene and are also 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 \textit{GFPT1} and \textit{UAPI} were increased by over 2-fold in both LNCaP and VCaP cells (Fig. 2A and 2B). The up-regulation of GFPT1 and UAPI was confirmed at the protein level in both cell lines (Fig. 2D and 2E).

To determine whether the hormone dependent up-regulation of the HBP enzymes is a feature found preferentially in cancer cell-lines, we used RWPE-1 cells.
Glycosylation regulates the stability of c-MYC 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 up-regulation 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 IC₅₀(18)) caused ~50% decrease in the viability in both cell lines (Fig. 3A and 3B). In addition, targeting OGT with siRNA reduced the growth rate of androgen receptor positive prostate cancer cells (Suppl. Fig. 1A).

OGT inhibitor decreases the expression of genes associated with DNA replication and cell cycle

The activity of OGT has been reported to be of high importance for the growth of cancer cells, while no study has attempted to assess the mechanism of action in an
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unbiased way(6, 11-13, 26, 27). However, effects on the activity of transcription factors through targeting OGT have previously been reported(11, 26). Consequently, to understand how OGT exerts its functions in prostate cancer cells, we utilized expression arrays. In order to minimize confounding effects from apoptosis, we selected a dose that caused maximally 20% decrease in cell viability after 48 hours of treatment. LNCaP cells were treated with OGT inhibitor or siRNAs targeting OGT (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, while siOGT reduced the levels of OGT mRNA by 80% (Fig 3C). Biological triplicate samples were analyzed by expression arrays and data was processed using Jxpress(28) to produce lists of up- and down-regulated genes from each treatment and time point (Suppl. Table 2). We first evaluated the expression profiles after treating cells with siRNAs targeting OGT. Strikingly, MGEA5, a gene encoding for O-GlcNAcase, was the top-most down-regulated gene (after OGT) at 24 hours (Suppl. Table 2 and Suppl. Fig. 1B). 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 five most up- or down-regulated genes from 24 hours time point for validation with qPCR. We observed 1.5- to 5-fold increase in the levels of DDIT3, AKR1C2, ARHGEF2, AKR1C4 and FAM129A (Fig. 3D). Next we analyzed the expression levels of the five most down-regulated genes CDC2,
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c11orf82, ATAD2, MCM10 and ASF1B, all of which, except for c11orf82, were
down-regulated by over 50% at a 24-hour time point. In order to understand the
significance of the altered gene expression profile, we performed a pathway
enrichment analysis with DAVID(30, 31) gene ontology enrichment tool (Suppl.
Table 2). This approach revealed a consistent down-regulation 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 down-regulated genes common to both time points in clinical samples using data
published in Taylor et al(32). Interestingly, genes down-regulated by OGT inhibitor
were over-expressed in the prostate cancer tissue of patients with metastatic prostate
cancer (Fig. 3E).

Inhibition of OGT de-stabilizes c-MYC in prostate cancer cells

OGT is a known epigenetic regulator(6) and we wanted to assess if OGT inhibitor
induced changes in the transcriptome are mediated by specific transcription factors.
We therefore took the 200 most down-regulated genes common to both time points
and uploaded them into the Ingenuity Pathway Analysis tool (IPA, Ingenuity®
Table 2). 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 to the expression levels of OGT in a large
prostate cancer gene expression array dataset(32) and found that c-MYC is tightly co-
Glycosylation regulates the stability of c-MYC expressed with OGT (Suppl. Fig. 2A). 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 neighbour associations with other factors as reported in the REACTOME. By these criteria c-MYC was the sole transcription factor linked to the network (Suppl. Fig. 2C).

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 three 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 and found 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 of OGT for 96 hours (Suppl. Fig. 3A). 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 (Suppl. Fig. 3B and 3C).

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
Glycosylation regulates the stability of c-MYC demonstrated in a range of cancers and amplification of the MYC locus has also been associated with poor prognosis in prostate cancer patients\(^{35}\). We speculated that OGT overexpression might associate with \(c\)-MYC copy number variation in the clinical setting to increase signalling via c-MYC oncogene in the lethal prostate cancer. The copy number status of MYC has been determined for 1306 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 co-expression 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 utilized 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 up-regulation 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, 1C 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-2F), 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 results 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 shRNA in mouse xenograft models decreases the growth of tumours(11, 13, 26). We found that the protein level expression of OGT correlates with high GS 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
Glycosylation regulates the stability of c-MYC progression (Suppl. table 2). 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 post-translational 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). By contrast, the OGT inhibitor we used here reduced the protein level of c-MYC without impacting on c-MYC transcript expression (Fig. 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 non-glycosylated pools of this important oncogene.

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

H.M.I. designed and performed the experiments, analysed data and drafted the manuscript. S.M. stained and scored tissue samples and performed statistical analysis on the clinical data. I.G. performed real-time PCR on patient samples and analysed the data. M. J. S. and M. C. T. stained tissue samples and analysed the clinical data. V. B., A. S. and T. S. assembled the clinical sample collections. I. G. M. initiated the study and contributed to the design of the experiments, data interpretation and the drafting of the manuscript. All authors contributed editing and preparing the final manuscript.
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References

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Tables

Table 1. Clinical parameters of OGT staining. Samples were analyzed based on the staining intensities (Int) shown in figure 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 Legends

Figure 1. Hexosamine biosynthetic pathway is up-regulated 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 five housekeeping genes (GAPDH, TBP, ALAS1, β-actin and 18S) and then to normal tissue. The significance of the expression was evaluated by paired samples T-test, *<0.05, **<0.01. B) Evaluation of OGT staining in localized prostate cancer. OGT staining was classified into four 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 biochemical relapse against post-operative time. The probability of biochemical relapse–free survival decreased in a dose-dependent manner with increasing OGT expression (P < 0.0001, across all intensities).

Figure 2. Androgen receptor regulates the expression of hexosamine biosynthetic pathway 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 (10nM R1881(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 is obtained from two biological replicates and for RWPE-1 cells from two technical replicates. D-F) Protein lysates were harvested at the indicated time points after R1881 stimulation and blotted.
Glycosylation regulates the stability of c-MYC for the proteins of interest. The intensity of WB 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 three 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 standard error of mean is shown. C) LNCaP cells were treated either with siRNA targeting OGT or with a low dose of OGT inhibitor causing 20% decrease in the viability after 48 hours of treatment. RNA was harvested in biological 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 was quantile normalised using Bead Studio and differentially expressed genes were identified using the JExpress software package. The five most up-regulated genes (*DDIT3*, *AKR1C2*, *ARHGEF2*, *AKR1C4*, *FAM129A*) or down-regulated genes (*CDC2*, *c11Orf82*, *ATAD2*, *MCM10*, *ASF1B*) from cells treated with OGT inhibitor for 24 hours were selected for validation of the data with qPCR. Expression of *MYC* mRNA is 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 down-regulated genes common to both time points after treatment with OGT inhibitor were compared to 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
Glycosylation regulates the stability of c-MYC into metastatic disease and compared these to the cases without prostate cancer (PAN) based on the clinical data described in Supplementary Table 1 of Taylor et al (32).

**Figure 4. OGT activity is required for c-MYC stabilization.** A) LNCaP, VCaP and PC3 cells were treated with a concentration gradient of OGT inhibitor for 48 hours and harvested for WB. RL2 antibody was used to assess the levels of O-linked glycosylation in cell lysates. Densitometric analysis of O-GlcNAc levels was performed on the entire lane as previously described (48) and normalized to the intensity of actin. The value for the vehicle treated condition was set to one and the O-GlcNAc signal in each treatment condition is given under the blot as a proportion of the vehicle signal. B) Evaluation of OGT staining and MYC alterations in prostatectomy samples. Samples were analyzed based on staining intensities for OGT shown in figure 1B while fluorescence *in situ* hybridization of MYC for this patient cohort has been reported previously(35). The classifications listed are ‘normal’ (no ploidy change), ‘poly’ (gene copy >2, ratio 1), ‘gain’ (ratio 1-2) and amp (ratio>2). We found statistically significant co-expression between OGT expression and MYC amplification (p=0.0012). C) Correlation between the mRNA level expression of OGT and c-MYC, OGT and AR and OGT and ERG in prostate biopsies (n=17). In each case, both normal and cancerous tissues were collected. The expression of the genes of interest was normalized to five housekeeping genes (GAPDH, TBP, ALAS1, β-actin and 18S) and then to normal tissue.

**Figure 5. Hexosamine biosynthetic pathway (HBP).** We used clinical gene expression data of up-regulated transcripts in localized prostate cancer(20) and ChIP-
Glycosylation regulates the stability of c-MYC

seq data on AR(2) and ERG(21) to identify a pathway that is over-expressed in clinical setting, targeted by AR and ERG in vitro and integrates metabolic flux. HBP requires several key metabolites, including glucose, glutamine, Acetyl-CoA and UTP, which makes this pathway capable of sensing overall energy status of the cell. The flux through HBP is highlighted with a grey arrow. OGT utilizes UDP-GlcNAc as a substrate to modify target proteins to regulate their activity, highlighted here as stabilization of c-MYC. GLUT1, Glucose transporter 1; HK1/2, Hexokinase-1/2; PFK1/2, Phosphofructokinase 1/2; ACACA, Acetyl-CoA carboxylase 1; FASN, Fatty acid synthase; NUDT9, nudix (nucleoside diphosphate linked moiety X)-type motif 9; GUCY1A3; guanylate cyclase 1, soluble, alpha 3; CANT1, calcium activated nucleotidase 1; GFTP1, glutamine--fructose-6-phosphate transaminase 1; GNPNAT, glucosamine-phosphate N-acetyltransferase 1; PGM, phosphoglucomutase; UAP1, UDP-N-acetylglucosamine pyrophosphorylase 1; OGT, O-linked N-acetylglucosamine (GlcNAc) transferase; SLC35A3 solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter). G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-BP, fructose 1,6-bisphosphate; GlcN-6-P; glucosamine-6-phosphate; GlcNAc-6-P, N-acetylglucosamine 6-phosphate; GlcNAc-1-P, N-acetylglucosamine 1-phosphate; UDP-GlcNAc, UDP-N-acetylglucosamine.
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Figure 1.
Figure 2.
Figure 3.

A and B: Graphs showing viability normalized to veh. (%)

C: OGT mRNA levels

D: OGT inhibitor for 24 hours

E: Heatmap of gene expression data.
Figure 4.

A

- LNCap
- VCap
- PES

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Densitometry:
- LNCap: 1.00, 0.96, 0.81, 0.72, 0.73, 0.66
- VCap: 1.08, 1.12, 0.81, 0.74, 0.49
- PES: 1.01, 0.79, 0.77, 0.61, 0.64

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O-GlcNAc transferase integrates metabolic pathways to regulate the stability of c-MYC in human prostate cancer

Harri Mikael Itkonen, Sarah Minner, Ingrid J Guldvik, et al.

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