Ubiquitin-specific protease 2a (USP2a) is overexpressed in almost half of human prostate cancers and c-Myc is amplified in one third of these tumor types. Transgenic MYC expression drives invasive adenocarcinomas in the murine prostate. We show that overexpression of USP2a downregulates a set of microRNAs that collectively increase MYC levels by MDM2 deubiquitination and subsequent p53 inactivation. By establishing MYC as a target of miR-34b/c, we demonstrate that this cluster functions as a tumor suppressor in prostate cancer cells. We identify a distinct mRNA signature that is enriched for MYC-regulated transcripts and transcription factor binding sites in USP2a overexpressing prostate cancer cells. We demonstrate that these genes are associated with an invasive phenotype in human prostate cancer and that the proliferative and invasive properties of USP2a overexpressing cells are MYC-dependent. These results highlight an unrecognized mechanism of MYC regulation in prostate cancer and suggest alternative therapeutic strategies in targeting MYC.

**SIGNIFICANCE:** The deubiquitinating enzyme USP2a has previously been shown to be oncogenic, overexpressed in almost half of human prostate adenocarcinomas, and prolongs the half-life of targets such as fatty acid synthase, MDM2, and cyclin D1. Here, we highlight a new mechanism by which USP2a enhances MYC levels through the modulation of specific subsets of microRNAs in prostate cancer, suggesting alternative therapeutic strategies for targeting MYC.

**ABSTRACT**

Ubiquitin-specific protease 2a (USP2a) is overexpressed in almost half of human prostate cancers and c-Myc is amplified in one third of these tumor types. Transgenic MYC expression drives invasive adenocarcinomas in the murine prostate. We show that overexpression of USP2a downregulates a set of microRNAs that collectively increase MYC levels by MDM2 deubiquitination and subsequent p53 inactivation. By establishing MYC as a target of miR-34b/c, we demonstrate that this cluster functions as a tumor suppressor in prostate cancer cells. We identify a distinct mRNA signature that is enriched for MYC-regulated transcripts and transcription factor binding sites in USP2a overexpressing prostate cancer cells. We demonstrate that these genes are associated with an invasive phenotype in human prostate cancer and that the proliferative and invasive properties of USP2a overexpressing cells are MYC-dependent. These results highlight an unrecognized mechanism of MYC regulation in prostate cancer and suggest alternative therapeutic strategies in targeting MYC.

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**multiple miRNAs and causes widespread reprogramming of the miRNA network, which has been found to directly contribute to tumorigenesis (4, 6, 16).**

Deubiquitinating enzymes represent one of the largest families of enzymes responsible for regulating proteins through the ubiquitin–proteasome system (17). Specific deubiquitinating enzymes regulate the stability and function of critical cellular factors such as MYC, p53, cyclin D1, and MDM2–MDMX (7, 18–21). Ubiquitin-specific protease 2a (USP2a) is a deubiquitinating enzyme that regulates the p53 pathway by targeting MDM2 (22). It also recognizes fatty acid synthase and cyclin D1 and modulates and prevents their proteasomal degradation (21–23). USP2a is overexpressed in almost half of human prostate adenocarcinomas, enhances tumorigenicity of prostate cancer cells in vitro and in vivo, and confers resistance to apoptosis induced by chemotherapeutic agents (24). Here we show that USP2a mediates suppression of the miRNA cluster miR-34b/c and that the consequent upregulation of MYC is critical for the tumorigenic potential of prostate cancer cells. Importantly, we show that overexpression of USP2a and downregulation of its target miR-34a/b through the modulation of the MDM2–p53 axis are associated with an invasive phenotype in prostate tumor cells. This is the first example of a mechanistic link between deubiquitination and miRNA expression, which in turn impacts the activity of MYC. These findings suggest that MYC, a driver of as many as one third of human prostate cancers, may be targeted by USP2a or miR-34b/c.

**INTRODUCTION**

The c-myc proto-oncogene is a transcription factor that plays a key role in regulating numerous cellular processes including metabolism, development, apoptosis, cell proliferation, and differentiation. Deregulated expression of MYC has been described in many human malignancies (including colon, breast, and prostate cancer) and plays a central role in their genesis (1). In prostate cancer, MYC seems to be a key player in disease progression and the presence of myc gene amplification (in up to 30% of cases) is associated with advanced histologic grade and worse prognosis (2). Transgenic mice expressing human MYC in the mouse prostate develop murine prostatic intraepithelial neoplasia followed by invasive adenocarcinoma and display a defined myc gene expression signature (3). Transcriptional regulation, posttranscriptional regulation, and ubiquitination appear to be important nodes in this MYC-driven network (4–8).

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, small noncoding RNAs that act as post-transcriptional regulators of gene expression. They primarily bind to the 3′ UTR of target transcripts leading to mRNA degradation or translational repression. Aberrant expression of miRNAs has been observed in human cancers (9, 10), where they can function as either tumor suppressor genes or oncogenes (11). Regulation of gene activity by miRNAs is critical to both normal cellular function and tumorigenesis. Recent studies have identified several miRNAs as regulators of MYC (12–15). Conversely, MYC regulates multiple miRNAs and causes widespread reprogramming of the miRNA network, which has been found to directly contribute to tumorigenesis (4, 6, 16).
RESULTS
USP2a Overexpression Downregulates miR-34b/c in Prostate Cells

To assess alterations in miRNA expression associated with USP2a overexpression, a curated set of prostate-specific miRNAs \((n = 51)\) \((10, 25, 26)\) was quantitated after overexpression of either USP2a^WT or USP2a^MUT \((C276A\text{\ and\ } H549R)\) in immortalized prostate epithelial cells \((\text{iPrEC})\) \((24, 27)\) (Fig. 1A). iPrEC–USP2a^WT cells exhibit an altered miRNA expression profile relative to the empty vector control and iPrEC–USP2a^MUT \((\text{Fig. 1B and Supplementary Fig. S1})\) characterized by significant and WT-specific downregulation of miR-98, the miR-34b/c cluster, and Let-7c and upregulation of miR-18a, miR-19a, and miR-20a. To validate the miRNA signature in cancer cells, the USP2a^WT-deregulated miRNAs were quantified in the androgen dependent prostate cancer cell line LNCaP. As observed in iPrEC, transfection with exogenous USP2a^WT triggers downregulation of miR-34b/c, miR-98, and let-7c expression levels, whereas miR-18a, 19a, and 20a are significantly induced when compared with USP2a^MUT and empty vector controls (Fig. 1C). Conversely, siRNA-mediated inhibition of endogenous USP2a expression increases miR-34b/c, miR-98, and let-7c expression by approximately 5-fold (Fig. 1D).

USP2a^WT-Deregulated miRNAs Lead to Increased MYC Expression

\textit{In silico} predictive models indicate that the whole set of USP2a-downregulated miRNAs potentially target \textit{c-myc} (Supplementary Fig. S2). To verify whether MYC levels are actually affected by USP2a activity, we assayed c-Myc protein levels in control, USP2a^WT, and USP2a^MUT iPrEC cells. Figure 2A shows that overexpression of USP2a^WT, but not

Figure 1. USP2a overexpression modifies the microRNA expression profile of prostate cells. \textbf{A}, analysis of USP2a mRNA and protein expression in empty vector (Vector), USP2a^WT, and USP2a^MUT iPrEC transfectants. \textbf{B}, prostate-specific microRNA expression profiling color heat-map carried out by TaqMan quantitative reverse-transcription PCR assay in the indicated iPrEC cells. Evaluation of USP2a protein expression and miRNA level analysis performed in LNCaP cells on \((\text{C})\) transfection with empty vehicle (Ve), pCDNA3 (Vector), pUSP2a^MUT, or pUSP2a^WT; and \((\text{D})\) incubation with empty vehicle (Ve), control (siControl), or specific USP2a (siUSP2a) silencing oligonucleotides. All data represent mean ± standard deviation of at least three independent replicates. \(P\) values: \(* P < 0.05\), \(** P < 0.01\), \(*** P < 0.001\). See also Supplementary Figure S1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot.
USP2a<sup>MUT</sup>, enhances MYC protein expression approximately 4-fold compared with parental cells (upper panel) and a corresponding increase in MYC-specific transcription target gene expression (Fig. 2A, bottom panel). However, if MYC is silenced in USP2a<sup>WT</sup> iPReC cells (Fig. 2B), its target gene expression (including the MYC-regulated oncogenic miR-17-5p cluster, miR-18a, miR-19a, and miR-20a) (6) is only partially silenced. This is also applicable to other experimental models, because a dose-response induction of USP2a<sup>MUT</sup>, but not USP2a<sup>WT</sup>, in LNCaP cells triggers the progressive upregulation of MYC along with the subset of MYC canonical target genes (Supplementary Fig. S3A). Indeed, MYC silencing in USP2a<sup>WT</sup>-overexpressing transfecitants partially reverts the USP2a-induced upregulation of the oncogenic miR-17-5p cluster expression (Supplementary Fig. S3B), similar to what is observed in primary iPReC cells.

The predicted annealing of miR-34b/c, miR-98, and let-7c to the 3’ UTR of the human <i>c-myc</i> transcript is schematized in Supplementary Figure S4A. Regulation of MYC by the miR-98/Let-7 family has previously been described (15), and both miR-98 and let-7c overexpression are able to reverse USP2a-driven MYC induction (Supplementary Fig. S4B). We then focused on MYC regulation by the miR-34b/c cluster. We first assessed whether MYC upregulation was dependent on miR-34-b/c deregulation in iPReC cells. Exogenous administration of increasing concentrations of synthetic miR-34b or miR-34-c molecules enhances miR-34b and miR-34c levels in a dose-dependent manner (Fig. 2C) while progressively down-regulating MYC protein expression (Fig. 2D). To determine whether this effect was direct, we assayed luciferase (LUC) reporter gene expression in iPReC cells transfected with a PGL3-primer vector carrying the <i>c-myc</i> 3’UTR cloned downstream of the LUC stop codon (28). The relative LUC activity levels were comparable in control and USP2a<sup>MUT</sup> iPReC clones but increase by approximately 6-fold in USP2a<sup>WT</sup> iPReC cells (Fig. 2E). Moreover, in wild-type cells, the high reporter gene activity is repressed in a dose-dependent manner after overexpression of either miR-34b or miR-34c (Fig. 2E). Consistent with the iPReC data, when USP2a<sup>WT</sup>-expressing LNCaP cells are transfected with exogenous miR-34b/c, MYC protein expression is impaired (Supplementary Fig. S5A). Similarly, MYC protein is depleted by USP2a silencing (siUSP2a) in LNCaP cells (Supplementary Fig. S5B, top panel) and restored by miR34b/c knockdown in infected cells (Supplementary Fig. S5B, bottom panel).

To verify whether the <i>c-myc</i> transcript is a direct target of USP2a-regulated miRNAs, a miR-IP assay was performed. Cells were cotransfected with expression vectors for HA-tagged AGO1 and the indicated miRNA mimics molecules (Fig. 2F). The immunoprecipitated RNA was analyzed by quantitative reverse transcription–PCR (qRT-PCR) using specific primers for <i>c-myc</i> and normalized to SS rRNA. miR-98 and let-7c were included as <i>c-myc</i>-targeting positive controls. Relative to input, <i>c-myc</i> was significantly (<i>P</i> < 0.002) enriched in the pulldown with miR-34b and miR-34c, thus demonstrating that <i>c-myc</i> miRNA is directly targeted by the miR-34 cluster. To further confirm the inverse relationship between MYC and miRNA expression, we queried the NCBI Gene Expression Omnibus database and identified two data sets in which measurements from the same patients were obtained for both mRNA and miRNA expression [GSE21034 and GSE21036 (29)]. Our analysis confirmed that on progression from a normal prostate to locally invasive prostate cancer, decreasing expression levels of hsa-miR-34b and hsa-miR-34c-5p are significantly associated with increasing MYC expression (both by t test and linear regression analyses; Supplementary Fig. S6).

Together these results strongly suggest that the increase in MYC protein caused by USP2a overexpression is mediated by the suppression of miR-34b/c. Thus, the <i>c-myc</i> transcript is directly targeted by the miR-34b/c cluster.

**USP2a Regulates the miR-34b/c Cluster by Directly Impairing the MDM2–p53 Pathway**

p53-mediated regulation of miR-34b/c and p53 binding sites in the miR-34b/c promoter region have been previously reported (26, 30, 31). In addition, the miR-34 family including miR-34b/c is regulated by p53 in ovarian tumors (26). However, because miRNAs are both temporal and spatial in their expression, we sought to verify through chromatin immunoprecipitation (ChIP) whether the p53 protein actually binds to the p53 putative binding sites (RE1 and RE2 regions) of the miR-34b/c promoter in prostate cancer cells. The human miR34b/c regulatory region was virtually divided into overlapping fragments (regions from A to O; Fig. 3A) and amplified in ChIP assays by specific oligonucleotide pairs in LNCaP cells. Figure 3B shows that the p53 transcription factor is recruited to the miR34b/c promoter in LNCaP cells, where it exclusively binds to the predicted RE2 region (fragment F) in a transcriptionally active status. The specificity of p53 binding to the miR-34b/c promoter was confirmed by ChIP after endogenous p53 silencing (Supplementary Fig. S7A), which was responsible for decreased miR-34b and miR-34c expression and associated with a downstream increase of MYC protein level (Supplementary Fig. S7B).

USP2a has been previously reported to directly bind and stabilize MDM2 (22). We therefore hypothesized that MDM2 can mediate the USP2a-driven regulation of miR34b/c cluster expression. As expected, we observed that USP2a levels strongly correlated with cellular levels of MDM2 (Supplementary Fig. S8). We next set out to demonstrate that altered MDM2 protein expression is responsible for the USP2a-driven regulation of miR-34b/c cluster expression in LNCaP cells (Fig. 3C). Indeed, ectopic induction of MDM2 reduces the expression of the miR-34b/c cluster. Similarly, MDM2 silencing triggers the overexpression of miR-34b/c (Fig. 3C). These results are supported by ChIP results that clearly demonstrate reduced p53 binding to the RE2 sequence (as seen with USP2a<sup>WT</sup> overexpression) in cells overexpressing MDM2 (Fig. 3D). Conversely, small interfering RNA-mediated silencing of MDM2 in the USP2a<sup>WT</sup> clone activates p53 and enhances its downstream recruitment to both the RE1 and RE2 containing sequences (Fig. 3D). Furthermore, treatment with Nutlin-3a (a specific MDM2 inhibitor) leads to dose-dependent activation of p53 in LNCaP cells with resultant upregulation of miR-34b/c and downregulation of MYC (Fig. 3E). Different p53 putative binding boxes have also been identified along the 5’ upstream regions (up to 50 kb) of both the miR-98 and let-7c genomic loci (Supplementary Fig. S9A). Both miRs are modulated by USP2a<sup>WT</sup> and are directly involved in the USP2a-mediated modulation of MYC expression; however, miR-98 and let-7c are not regulated by p53 in prostate cancer cells. In fact, direct and USP2a-mediated p53 inhibition (Supplementary Fig. S9B) together with siUSP2a and Nutlin-mediated p53 induction (Supplementary Fig. S9C) are both unable to affect
**Figure 2.** USP2a-dependent miRs deregulation modulates MYC expression. A, Western blot analysis of MYC protein level and evaluation of MYC specific transcription target genes carried out in USP2a overexpressing immortalized prostate epithelial cells (iPrEC) cells. B, evaluation of MYC protein level (left panel), MYC–regulated transcripts, and microRNAs (right panels) performed in empty vector iPrEC cells (Vector) and iPrEC–USP2aWT cells silenced for either green fluorescent protein or MYC expression. Analysis of miR-34b and miR-34c (C) and MYC expression levels (D) in iPrEC–USP2a WT cells after treatment with increasing concentrations of control (CTR), miR-34b, and miR-34c synthetic molecules. E, luciferase (LUC) reporter assay performed in empty vector (Vector), USP2aMUT, and USP2a WT iPrEC transfectants (top panel) and in USP2a WT iPrEC clone exposed to increasing concentrations of control (CTR), miR34b, and miR-34c synthetic molecules (bottom panel). F, isolation of target transcripts associated with miR-34b/c. Cells were cotransfected with expression vectors for HA-tagged AGO1 and the indicated microRNA mimics and a control mimic. The immunoprecipitated RNA was analyzed by qRT-PCR using specific primers for c-myc and normalized to 5S rRNA and immunoprecipitate from control mimic. miR-98 and let7c, which are known to target c-myc, served as controls. Relative to input, c-myc was significantly (*P < 0.05, **P < 0.01, ***P < 0.001) enriched in the pulldown with miR-34b and miR-34c. All data represent mean ± standard deviation of at least 3 independent replicates. P values: *P < 0.05, **P < 0.01, ***P < 0.001. See also Supplementary Figures S2 to S5. WB, Western blot.
miR-98/let-7c expression or trigger p53 recruitment to its response elements in miR regulatory regions in LNCaP cells (Supplementary Fig. S9D). Our data suggest that, in prostate cancer cells, USP2a-mediated induction of MDM2 and subsequent degradation of p53 impedes the transcription of the miR-34b/c miRNA cluster.

To verify the biochemical effect of USP2a expression on the MDM2-p53 pathway and the subsequent impact on MYC protein expression, we carried out a time-course experiment to measure the levels of USP2a, MDM2, p53, and MYC and miR-34b/c at different points after USP2a WT overexpression in LNCaP cells (Fig. 4). USP2a expression is first detectable at 12 hours posttransfection and is sustained during the first 36 hours. Subsequently, MDM2 is upregulated (24–36 hours posttransfection) and p53 is downregulated (36–60 hours posttransfection). Although the decrease in miR-34b/c expression occurs as early as 12 hours after USP2a transfection, the final effect on MYC overexpression is evident after 60 to 72 hours. We confirmed these results by using a prostate cell line (PC3) devoid of p53. When we restore wild-type p53 presence under USP2a expression, we observe a decrease in MYC expression similar to LNCaP. Conversely, nondegradable p53 mutants (Δ13-19 or 22Gln/23Ser) and USP2a coexpression lead to an upregulation of MYC expression (data not shown). Taken together, these results suggest that USP2a regulates MYC expression through the MDM2–p53 axis.

mRNA Signature of USP2a-Overexpressing Cells

To characterize the molecular signature associated with USP2a overexpression, we next analyzed differential gene expression in iPrEC transfected with USP2a WT and USP2a MUT using the Affymetrix Human Genome U133 Plus 2.0 Array (Fig. 5). Empty vector was used as a control (EV). Direct comparison of the three distinct groups of samples (WT, MUT, EV) revealed robust differential expression of distinct sets of genes and cellular pathways associated with the expression of USP2a WT. A total of 789 genes were differentially expressed between USP2a WT and USP2a MUT; 848 genes were differentially expressed between USP2a WT and EV; 54 genes were differentially expressed between USP2a MUT and EV (P < 0.0001 after adjustment for multiple testing in all pairwise comparisons). USP2a overexpression was associated with a global gene expression upregulation, and mRNA transcripts for several cancer-related genes such as c-myc were more highly expressed in the USP2a WT
stromal compartment. Interestingly, USP2aWT genes appeared to be strongly upregulated during local invasion of cancer cells, because they were most strongly enriched when locally invasive cancers were compared with prostatic intraepithelial neoplasia specimens (Fig. 5C).

**Targeting MYC in USP2a-Overexpressing Cells Suppresses Growth and Invasion**

USP2a modulation of MYC protein expression may have important biologic implications for the natural history of prostate cancer. We demonstrate that the proliferative capacity, cell viability, and clonogenic ability of LNCaP USP2a WT cells can be significantly impaired by targeting MYC expression. This can be achieved by direct silencing of MYC, by administration of Nutlin-3a, or by treating USP2a WT cells with synthetic miR-34b/c molecules (Supplementary Fig. S12).

Because enrichment of the genes upregulated by USP2aWT is observed during local invasion of prostate cancer (Fig. 5C), we next analyzed publicly available gene expression profiles of a number of distinct cancer invasion models. CAT-plot analysis revealed significant agreement between genes upregulated by USP2aWT and those upregulated on invasion in each model considered (P < 0.001). The strongest agreement was observed with migration efficiency/velocity in Boyden chamber assays using cell lines from prostate and other cancers (Fig. 6). Interestingly, in the prostasphere invasion model, the strongest agreement was observed between the USP2a signature and the genes differentially expressed between the “stellate” and “mass” phenotypes, whereas overlap was observed when the comparison involved the “round/branching” noninvasive phenotypes (data not shown). The “stellate” phenotype corresponds to invasive cancer cell lines, the “mass” phenotype to noninvasive cancer cell lines and transformed normal prostatic epithelial cells, and the “round/branching” phenotype to iPReCs and non-transformed lines. In agreement with the CAT-plot analysis, the

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**Figure 4.** USP2a regulates MYC expression through the MDM2–p53 axis. **A,** kinetics profile (0–72 hours) of protein levels in LNCaP cells after pUSP2aWT (purple bars) overexpression or empty vector (green bars). Representative blots are shown at each set point for the specific protein (upper panel) and actin levels (lower panel). **B,** miR-34b/c expression profile under the same conditions depicted in A.
Deubiquitinating enzymes can prevent destruction of protein substrates before proteasomal degradation. USP2a deubiquititates the antiapoptotic proteins fatty acid synthase, the ubiquitin ligase MDM2, and cyclin D1. USP2a is overexpressed in approximately 40% of human prostate adenocarcinomas (24) and exhibits oncogenic behavior both in vitro and in vivo. In addition, USP2a silencing in several human cancer cell lines results in apoptosis.

Oncogenic activation of *c-myc* is one of the most frequent events in human malignancy in general and prostate cancer in particular. *c-myc* is a master regulatory gene that can globally reprogram cells to proliferate or undergo apoptosis through induction or repression of transcription. There are a number of molecular mechanisms hypothesized for the oncogenic function of *MYC* (1, 37–40). Importantly, this study highlights a previously unrecognized mechanism of MYC regulation establishing a novel link between deubiquitination, miRNA de-regulation, and the p53/MYC regulatory network. Gene set enrichment analysis of microarray expression in human tumors are associated with prostate tumor progression. Specifically, we show that the proliferative capacity, cell viability, clonogenic ability, and invasive properties of USP2aWT overexpressing cells are significantly impaired by targeting MYC expression. Although novel therapeutics to target MYC-dependent tumors have been developed (41–43), these are generally not effective. This study provides evidence that, at least in prostate cancer, miRNA expression patterns correlate strikingly with disease progression and response to therapy (44). However, the biological roles of only a very limited number of miRNAs have been examined to date. The identification of new miRNA targets by USP2a overexpression is a promising approach that may provide a wealth of new targets for therapeutic intervention.

**DISCUSSION**

In vitro invasion test demonstrates that USP2aWT overexpression increases the invasive ability of iPcEC when compared with control or USP2aMUT-transfected cells, and, when silenced in an LNCaP experimental model, reduces invasion by almost 2-fold (Fig. 6C). miR-34c shows concordant downregulation. See also Supplementary Figures S9 and S10.

**Figure 5.** USP2a overexpression is associated with a discrete mRNA signature that is enriched for MYC-regulated transcripts. **A**, Venn diagram of differentially expressed genes in immortalized prostate epithelial cells (iPrEC) transfected with USP2aWT and USP2aMUT and empty vector (EV) control. Nominal adjusted *P* value < 0.0001; results are for all nonredundant NCBI entrez gene identifiers available on Affymetrix U133 Plus 2.0 Array. Red, upregulated genes; green, downregulated genes. **B**, functional annotation analysis for genes differentially expressed between USP2aWT and USP2aMUT. Genes were ordered by moderated t-statistics obtained from USP2aWT and USP2aMUT contrast, whereas functional gene sets were obtained from different databases (see Supplementary Microarray Methods and Results sections). **C**, enrichment analysis of microarray expression in human tumors significantly affect the relative prostate invasive ability (Fig. 6C). miR-34b/c seems to be involved in the USP2a-mediated invasiveness. Genes were ordered by moderated t-statistics obtained from the Wilcoxon rank-sum test.
Figure 6. USP2a overexpression increases the invasive ability of prostate cells. CAT plot showing the agreement between USP2a and invasion gene expression signatures. Genes upregulated by wild-type USP2a transfection as compared with mutant USP2a showed significant overlap with genes expressed on invasion in a number of distinct laboratory models. On the y-axis is shown the proportion of genes in common; on the x-axis is shown the number of top ranking genes considered. The black line corresponds to proportions of common genes by chance; gray shades around this black line correspond to decreasing probabilities of agreement by chance (0.05, 0.01, and 0.001). Therefore, CAT curves in the top white area of the graph have a probability of agreeing by chance of $P < 1E-03$. In the figure, genes were ranked by decreasing $t$-statistic as obtained from our linear model analysis. Each line represents a different pairwise comparison between differential gene expression between wild-type and mutant USP2a compared with differential gene expression between: A, (A) fast and slow migrating cell lines from various cancer types in Boyden assays (red line); (B) fast (PC3) and slow (LNCaP) migrating prostate cancer (PCA) cell lines in Boyden assays (blue line); (C) mouse male and female urogenital sinuses at gestational day 17 ($\pm$ 0.5) (green line); (D) Matrigel invasive and noninvasive prostaspheres (purple line); (E) laser capture microdissected epithelial cells from locally invasive prostate cancer and prostatic intraepithelial neoplasia (PIN) (orange line); B, (A) fast and slow migrating cell lines from various cancer types in Boyden assays (red line); (B) fast (PC3) and slow (LNCaP) migrating prostate cancer cell lines in Boyden assays (blue line); (C) mouse male and female urogenital sinuses at gestational day 17 ($\pm$ 0.5) (green line); (D) Matrigel invasive and noninvasive prostaspheres (purple line); C, evaluation of relative invasive ability performed in iPrEC clones and in LNCaP cells by ECMatrixTM Invasion Chamber. The iPrEC–USP2aWT transfectant has been also treated with miR synthetic molecules (mimic), whereas USP2a-silenced LNCaP cells underwent further treatment with specific antagonists (KO molecules) before invasion assessment. Data represent mean ± standard deviation of 4 independent replicates. $P$ values: * $P < 0.05$, ** $P < 0.01$.

elucidated in cancer cells. The downregulation of miR-34b/c expression that results from USP2a overexpression suggests a direct role of the miR-34b/c cluster as a potential tumor suppressor in prostate cancer. Furthermore, the regulation of miRNAs by USP2aWT is the first example of the ubiquitin–proteasome pathway playing a role in miRNA biology. We outline our proposed model for this mechanism in Figure 7.

Several studies have demonstrated the use of developing specific inhibitors of ubiquitin ligases and deubiquitinating enzymes as valuable therapeutic strategies against cancer (45, 46). Our results further underscore the importance of these therapeutic strategies and suggest that USP2a inhibitors may offer a potentially viable means of targeting MYC-dependent prostate cancer.

**METHODS**

**Cell Culture**

Empty vector (Vector), wild-type (USP2aWT), and mutant (USP2aMUT) stable clones (previously established by infecting
iPrEC [23, 24] were grown in specific PrEBM medium; Cambrex) and selected in 1.6 μg/mL puromycin. Human prostate adenocarcinoma LNCaP and PC3 cells were obtained from the American Type Culture Collection (ATCC) and grown in RPMI-1640 medium (Invitrogen, Life Technologies) containing 10% fetal bovine serum (GIBCO-Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Cell lines were purchased from ATCC in 2006. Both LNCaP and PC3 cells have been periodically tested (every 3 months) for cell morphology, growth rate, colony-forming ability, and gene expression of both p53 and AR. Moreover, cells have undergone a contamination test for excluding mycoplasma presence by Hoechst staining and PCR-based exogenous DNA detection.

**Cell Transfection and Luciferase Reporter Assay**

Transient transfection experiments were carried out by seeding cells in 60-mm Petri dishes in complete medium (2 × 10⁵ cells/plate). Transfection was performed 24 hours after plating using Lipofectamine 2000 reagent in Optimum medium (Invitrogen) for siRNA oligonucleotides (100–500 nM) and synthetic/knockdown miR molecules (10–50 nM) and by JetPei reagent (PloyPlus-Transfection) in complete medium for expression vectors (0.5–5 μg) and LUC-reporter plasmids. Evaluation of protein, mRNA, and miRNA expression levels was performed by harvesting cells at 24-hour intervals (24–120 hours) after transfection. For LUC reporter assays, cells were cotransfected with the pGL3-rtLuc UTR vector (1 μg) and PEQ-176 (expressing the control β-galactosidase enzyme, 1 μg); scrambled (CTR), or increasing concentrations of synthetic miR sequences were added to the transfection mix for iPrEC-USP2a cells. Forty-eight to 96 hours later, cells were rinsed with cold phosphate-buffered saline, resuspended in cell lysis buffer (Promega), and incubated for 10 minutes at room temperature. Insoluble material was spun down, and LUC activity was quantified by a commercially available kit (Promega) on a TD-20E luminometer (Turner Biosystems). Luciferase values were normalized to β-galactosidase expression. All sequences of siRNA are reported in Supplementary Table S1.

**Western Blot**

Cell lysis was performed on ice for 30 minutes in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA) supplemented with phosphatase inhibitors (1 mM PMFS, 1 μg/mL aprotinin, leupeptin, pepstatin). Equal amounts of total protein extracts (20–80 μg) were resolved by 10%, 12%, or 15% denaturing sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred for 4 hours to polyvinylidene difluoride membrane. Membranes were blocked in 5% milk–phosphate-buffered saline–0.05% Tween 20 for 1 hour and incubated overnight with the specific primary antibodies. Secondary antibodies were horseradish peroxidase-conjugated (Santa Cruz), and ECL reagent (Amersham, GE Healthcare) was used for chemiluminescence detection.

**RNA Isolation and qRT-PCR for mRNA Analysis**

Total RNA was extracted using Trizol Reagent (Invitrogen). RNA quantity and integrity were assessed using a NanoDrop Spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. For cDNA synthesis, 2 μg of total RNA was reverse-transcribed with random primers by a Moloney murine leukemia virus reverse transcriptase kit (M-MLV RT kit; Invitrogen), according to the manufacturer's instructions. Semiquantitative RT-PCR was performed with the Applied Biosystems Taq enzyme (Applied Biosystems by Life Technologies) using 2 μL of cDNA. Polymerase chain reaction conditions were as follows: one cycle at 94°C (5 minutes); 23–35 cycles at 94°C (30 seconds), 58°C (40 seconds), 72°C (40 seconds); and one cycle at 72°C (7 minutes). Polymerase chain reaction products were run on a 2% agarose gel and visualized with ethidium bromide. Gene transcript expression was normalized to glyceraldehyde-3-phosphate dehydrogenase levels. qRT-PCR was carried out with 1 μL of cDNA using SYBR Green master mix (Applied Biosystems) and analyzed on a StepOne Plus Real Time PCR Detection System (Applied Biosystems). All reactions were run in triplicate and the relative abundance of specific mRNA levels was calculated by normalizing to both glyceraldehyde-3-phosphate dehydrogenase and aldolase A expression using the 2^-ΔΔCt method (47). All sequences of oligonucleotide primers are reported in Supplementary Tables S2–3.

**Immunoprecipitation of miRNA Targets**

PC3 cells (0.5 × 10⁵ cells) were cotransfected with 1 μg of HA-AQO1 (pIRES-FLAG/HA vector; Addgene) and 50 nM of miRNA mimics using Lipofoxamine 2000 (Invitrogen). After 2 days, cells were harvested using 400 μL lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40) containing freshly added RNaseOUT (Invitrogen) and Protease Inhibitor Cocktail (Roche). After centrifugation, a 50-μL aliquot of supernatant was taken as input for subsequent RNA extraction. The remaining supernatant was gently shaken with HA-beads (HA-probe Santa Cruz sc-7392) for 4 hours at 4°C in Spin Columns-Screw Cap (Pierce). The columns were drained, washed, and the retained beads were treated with 5 U DNaseI in NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.05% Nonidet P-40) for 10 minutes at 37°C, washed with NT2, and then treated with Proteinase K in NT2 plus 1% sodium dodecyl sulphate for 30 minutes at 55°C. Finally, the beads were resuspended in NT2 buffer and RNA was extracted using acid phenol-chloroform (Ambion-Applied Biosystems) to extract RNA. RNA was reverse-transcribed and analyzed with quantitative PCR for enrichment of c-myc miRNA.

**Chromatin Immunoprecipitation Assay**

Cells were washed with phosphate-buffered saline (supplemented with phosphatase inhibitors) and incubated for 10 minutes with 1% formaldehyde at room temperature. Formaldehyde crosslinking was stopped by adding glycine, pH 2.5 (125 mM final concentration), for 5 minutes at room temperature. Cells were scraped off the plates, lysed in sodium dodecyl sulphate lysis buffer (1% sodium dodecyl sulphate, 10 mM EDTA, 50 mM Tris-HCl, pH 8, plus protease inhibitor mixture), and sonicated to generate 500- to 2000-bp fragments. After centrifugation, the supernatant was diluted 10-fold in immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) and incubated at 4°C with Protein A/G beads (Pierce) (previously adsorbed with sonicated single-stranded DNA and bovine serum albumin). The cleared lysate was incubated

![Image](image-url)
overnight with specific antibody, and the immune complex was further precipitated with protein A/G. After centrifugation, the beads were washed and the antigen eluted with 1% dodecyl sulphate and 100 mM sodium carbonate. DNA-protein crosslinks were reversed by heating at 65°C for 4–5 hours, and DNA was phenol-extracted and ethanol-p precipitated. Amplification of specific regions on genomic immunoprecipitated chromatin was carried out by PCR with the Applied Biosystem Taq enzyme (Applied Biosystems) using 2 μL of DNA. Polymerase chain reaction conditions were as follows: one cycle at 94°C (5 minutes); 28 to 35 cycles at 94°C (30 seconds), 58°C (40 seconds), 72°C (40 seconds); and one cycle at 72°C (5 minutes). PCR products were run on a 2% agarose gel and visualized with ethidium bromide. Details of the in silico analysis of putative miRNA promoters and oligonucleotide primer sequences for the ChIP assay are outlined in Supplementary Information and Supplementary Tables S4–7.

**qRT-PCR for miRNA Analysis**

Analysis of miRNA expression was carried out on total RNA using real-time stem-loop reverse transcription–PCR according to the manufacturer’s protocol (Taqman miRNA assays are available as assays on demand; Applied Biosystems). Briefly, 10 ng of total RNA was reverse-transcribed with specific miR stem-loop primer and subsequently amplified by miR-specific primers. qRT-PCR was done using a StepOne Plus Real Time PCR Detection System (Applied Biosystems). All reactions were run in triplicate and the relative abundance of specific miRs was calculated by normalizing to small nuclear RNAs using the 2−ΔΔCt method (47).

**In Vitro Cell Invasion Analysis**

Cell invasion ability was assessed using the Chemicon Invasion Chamber (Chemicon, Millipore), a tissue culture plate modified with insert containing an 8-μm pore size polycarbonate membrane over which a thin layer of ECMatrix is dried. Briefly, inserts were hydrated for 2 hours with 300 μL of warm serum-free media at 37°C with 5% CO². After carefully removing medium from the inserts, 500 μL of medium (PrEBM plus supplements and RPMI with 10% fetal bovine serum for iPrEC and LNCaP cells, respectively) were added to the lower chamber, whereas 300 μL of cell suspension (10⁶ cells/mL) of iPrEC clones in PrEBM and LNCaP cells in RPMI plus 2% fetal bovine serum (scControl or stUSP2a-treated before harvest) were added to the top chambers. Cells were allowed to invade for 48 hours (iPrEC) or 24 hours (LNCaP) at 37°C with 5% CO². After incubation, noninvasive cells were gently removed from the interior of the inserts with a cotton-tipped swab. Invasive cells on the lower surface of the membrane were stained by dipping inserts in the kit staining solution for 20 minutes and washing several times in water. After air-drying, stained cells were dissolved in 10% acetic acid (100–200 μL/well) and quantitated by reading OD at 560 nm.

**Statistical Analysis**

All data are presented as mean ± standard deviation calculated for 3 or more replicates. All statistical analyses were performed using an unpaired 2-tailed t test using SPSS Software Version 1, unless differently specified. P values < 0.05 were taken as statistically significant.

**Gene Expression Profiling and Functional Annotation Analysis**

Total RNA was extracted from iPrEC cells using Trizol (Invitrogen) purified using a DNase 1 (Qiagen) digestion step and further enriched using Qiagen RNaseasy columns. RNA quantity and integrity were assessed using a NanoDrop Spectrophotometer (Thermo Scientific) and a thin layer of ECMatrix is dried. Briefly, inserts were hydrated for 2 hours with 300 μL of warm serum-free media at 37°C with 5% CO². After carefully removing medium from the inserts, 500 μL of medium (PrEBM plus supplements and RPMI with 10% fetal bovine serum for iPrEC and LNCaP cells, respectively) were added to the lower chamber, whereas 300 μL of cell suspension (10⁶ cells/mL) of iPrEC clones in PrEBM and LNCaP cells in RPMI plus 2% fetal bovine serum (scControl or stUSP2a-treated before harvest) were added to the top chambers. Cells were allowed to invade for 48 hours (iPrEC) or 24 hours (LNCaP) at 37°C with 5% CO². After incubation, noninvasive cells were gently removed from the interior of the inserts with a cotton-tipped swab. Invasive cells on the lower surface of the membrane were stained by dipping inserts in the kit staining solution for 20 minutes and washing several times in water. After air-drying, stained cells were dissolved in 10% acetic acid (100–200 μL/well) and quantitated by reading OD at 560 nm.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

MYC Activation by USP2a-Mediated Modulation of MicroRNAs


MYC Is Activated by USP2a-Mediated Modulation of MicroRNAs in Prostate Cancer

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