c-Myc is activated via USP2a-mediated modulation of microRNAs in prostate cancer

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Running Title: c-Myc activation via USP2a-mediated modulation of microRNAs

Keywords: Ubiquitin-Specific Protease 2a (USP2a), c-MYc, microRNAs, prostate cancer

Financial Support: NCI RO1CA131945, PO1CA89021, P50CA90381, to ML and a gift from Nuclea Biomarkers to the laboratory of ML. NIH P30CA006973 to LM. European Community (EC) Active p53 Program to GB. Sabbatical research fellowship (CAPES #3665/10-0) and salary support are provided by Brazilian Government to SZ.

Disclosure of potential conflicts of interest: No conflicts of interest were disclosed. This publication reflects the authors’ views and not necessarily those of the European Community.

Word Count: 5009
Total number of Figures and Tables: 7

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ABSTRACT

Ubiquitin-Specific Protease 2a (USP2a) is over-expressed in almost half of human prostate cancers and c-Myc is amplified in a third of these tumour types. As a transgene c-Myc results in invasive adenocarcinomas in the murine prostate. We show that over-expression of USP2a, down-regulates a set of microRNAs (miRNAs) which collectively increase c-Myc, via Mdm2 de-ubiquitination and subsequent p53 inactivation. By establishing c-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, which is enriched for c-Myc-regulated transcripts and transcription factor binding sites in USP2a over-expressing 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 over-expressing cells are c-Myc-dependent. These results highlight an unrecognized mechanism of c-Myc regulation in prostate cancer and suggest alternative therapeutic strategies in targeting Myc.
SIGNIFICANCE

Previously it has been shown that deubiquitinating enzyme USP2a is oncogenic, is over-expressed in almost half of human prostate adenocarcinomas and targets Fatty Acid Synthase, Mdm2 and Cyclin D1 prolonging their half life. Here we highlight a new mechanism by which USP2a enhances c-Myc levels via the modulation of specific subsets of microRNAs in prostate cancer suggesting alternative therapeutic strategies for targeting Myc.
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 c-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, c-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 histological grade and worse prognosis\(^2\). Transgenic mice expressing human c-Myc in the mouse prostate develop murine prostatic intraepithelial neoplasia followed by invasive adenocarcinoma and display a defined Myc signature by gene expression profiling\(^3\). Transcriptional regulation, post-transcriptional regulation and ubiquitination appear to be important nodes in this network\(^4\)\(^-\)\(^8\).

miRNAs are evolutionarily conserved, endogenous, small non-coding RNAs that act as posttranscriptional regulators of gene expression. They primarily bind to the 3’UTR of target transcripts leading to mRNA degradation or translation repression. Aberrant expression of miRNAs has been observed in human malignancy\(^9\)\(^,\)\(^10\)\(^,\), 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 c-Myc\(^12\)\(^-\)\(^15\). Conversely, c-Myc regulates multiple miRNAs and causes widespread reprogramming of the miRNA network, which has been found to directly contribute to tumorigenesis\(^4\)\(^,\)\(^6\)\(^,\)\(^16\).
Deubiquitinating enzymes (DUBs) represent one of the largest families of enzymes responsible for regulating proteins via the ubiquitin-proteasome system\textsuperscript{17}. Specific DUBs regulate the stability and function of critical cellular factors such as c-Myc, p53, Cyclin D1 and Mdm2/Mdmx\textsuperscript{7,18-21}. USP2a is a DUB that regulates the p53 pathway by targeting Mdm2\textsuperscript{22}. It also recognizes Fatty Acid Synthase (FASN) and Cyclin-D1, modulating and preventing their proteosomal degradation\textsuperscript{21-23}. USP2a is over-expressed 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\textsuperscript{24}. Here we show that USP2a mediates suppression of the miRNA cluster miR-34b/c, and that the consequent up-regulation of c-Myc, is critical for the tumorigenic potential of prostate cancer cells. Importantly, we show that over-expression of USP2a and down-regulation of its target miRNA 34a/b via 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 de-ubiquitination and microRNA expression, which in turn impacts the activity of c-myc. These findings suggest that c-Myc, an important driver of as many as one third of human prostate cancers, may be targeted via USP2a or miRNAs 34b/c.

RESULTS

USP2a over-expression down-regulates miR-34b/c in prostate cells

In order to assess the alteration in miRNA expression associated with USP2a over-expression, a curated set of prostate-specific miRNAs (n= 51)\textsuperscript{10,25,26}, were quantitated following over-expression of either USP2a\textsuperscript{WT} or USP2a\textsuperscript{MUT} in
immortalized prostate epithelial cells (iPrEC)\textsuperscript{24,27} (Fig. 1a). iPrEC-USP2a\textsuperscript{WT} cells exhibit an altered miRNA expression profile relative to the empty vector control and iPrEC-USP2a\textsuperscript{MUT} (Fig. 1b and Supplementary Fig. 1), characterized by a significant and WT-specific down-regulation of miR-98, miR-34b/c cluster, and Let-7c, and parallel up-regulation of miR-18a, miR-19a and miR-20a. To validate the miRNA signature in cancer cells, the USP2a\textsuperscript{WT}-deregulated miRNAs were quantified in the androgen dependent prostate cancer cell line LNCaP. As observed in iPrEC, transfection with exogenous USP2a\textsuperscript{WT} triggers a down-regulation of miR-34b/c, miR-98 and let-7c expression levels, whereas miR-18a, 19a and 20a undergo a significant induction when compared to USP2a\textsuperscript{MUT} and empty vector controls (Fig. 1c). Conversely, the inhibition of endogenous USP2a expression, by siRNA, increases miR-34b/c, miR-98 and let-7c expression by ~5 fold (Fig. 1d).

**USP2a\textsuperscript{WT}-deregulated miRNAs lead to increased c-Myc expression**

The whole set of USP2a-downregulated miRNAs potentially target \textit{c-myc}, in \textit{in silico} predictive models (Supplementary Fig. 2). To verify whether c-Myc levels are actually affected by USP2a activity, we assayed c-Myc protein levels in control, USP2a\textsuperscript{WT} and USP2a\textsuperscript{MUT} iPrEC cells. Fig. 2a shows that over-expression of USP2a\textsuperscript{WT}, but not USP2a\textsuperscript{MUT}, enhances c-Myc protein expression about 4-fold compared to parental cells (upper panel), as well as its functionality, as c-Myc specific transcription target genes undergo a corresponding increase in USP2a\textsuperscript{WT} cells (Fig. 2a, bottom panel). However, if c-Myc is silenced in USP2a\textsuperscript{WT} iPrEC cells (Fig. 2b), its target gene expression (including the c-Myc-regulated
oncogenic miR-17-5p cluster, miR-18a, miR-19a and miR-20a, ref 6.) is only partially silenced. This is also applicable to other experimental models, as a dose-response induction of USP2aWT in LNCaP cells triggers the progressive up-regulation of c-Myc, along with the subset of its target genes, whereas USP2aMUT over-expression does not lead to any significant modulation (Supplementary Fig. 3a). Indeed, c-Myc silencing in USP2aWT over-expressing transfectants partially reverts the USP2a-induced up-regulation of the oncogenic miR-17-5p cluster expression (Supplementary Fig. 3b), similar to what observed in primary iPrEC cells.

The predicted annealing of miR-34b/c, miR-98 and let-7c to the 3'UTR of the human c-myc transcript is schematized in Supplementary Fig. 4a. Regulation of c-Myc by miR-98/Let-7 family has previously been described15, and both miR-98 and let-7c over-expression are able to revert the USP2a-driven c-Myc induction (Supplementary Fig. 4b). We then focused on c-Myc regulation by the miR-34b/c cluster. We first assessed whether c-Myc up-regulation was dependent on the miR-34-b/c deregulation in iPrEC cells. Exogenous administration of increasing concentrations of synthetic miR-34b or miR-34c molecule enhances miR-34b and miR-34c levels in a dose dependent manner (Fig. 2c), while progressively down-regulates c-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-promoter vector carrying the c-myc 3'UTR cloned downstream the LUC stop codon28. Relative luciferase activity displays comparable levels in control and USP2aMUT iPrEC clones, but increases by ~6 fold in USP2aWT iPrEC cells (Fig. 2e); moreover, in wild type cells, the high reporter gene activity is
repressed in a dose-dependent manner following over-expression of either miR-34b or miR-34c (Fig. 2e). In keeping with iPrEC data, when USP2aWT expressing LNCaP cells are transfected with exogenous miR-34b/c, c-Myc protein expression is impaired (Supplementary Fig. 5a). Similarly, c-Myc protein is depleted by USP2a silencing (siUSP2a) in LNCaP cells (Supplementary Fig. 5b, top panel), and restored by miR34b/c knock-down in infected cells (Supplementary Fig. 5b, bottom panel).

To verify whether the c-myc transcript is a direct target of USP2a-regulated microRNAs, a miR-IP assay was performed. Cells were co-transfected with expression vectors for HA-tagged AGO1 and the indicated miRNA mimic molecules (Fig. 2f). The immune-precipitated RNA was analyzed by qRT-PCR using specific primers for c-Myc, and normalized to 5S rRNA. miR-98 and let7c were included as c-Myc-targeting positive controls. Relative to input, c-Myc was significantly (p<0.002) enriched in the pull-down with miR-34b and miR-34c, thus demonstrating that c-myc mRNA is directly targeted by the miRNA-34 cluster. To further confirm the negative relationship between c-Myc and microRNA expression we queried the NCBI Gene Expression Omnibus database and identified two datasets in which measurements from the same patients were obtained for both mRNA and microRNA expression (GSE21034 and GSE21036, Taylor et al, Cancer Cell, 2010,). Our analysis confirmed that upon progression from 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. 6).
Together these results strongly suggest that an increase in c-Myc protein, when USP2a is overexpressed, is mediated by the suppression of miR-34b/c. Thus, the c-Myc transcript is directly targeted by the miRNA cluster, miR-34b/c.

**USP2a regulates the miR-34b/c cluster by directly impairing the Mdm2-p53 pathway**

p53-mediated regulation of miR-34b/c and the p53 binding sites in the miR-34b/c promoter region have been previously reported\(^{26,29,30}\). In addition, the miR-34 family including miR-34b/c is regulated by p53 in ovarian tumors\(^{26}\). However, as miRNAs are both temporal and spatial in their expression, we sought to verify through chromatin immunoprecipitation (ChIP) whether p53 protein actually binds to the putative 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. **Fig. 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 following endogenous p53 silencing (**Supplementary Fig. 7a**), responsible for decreasing miR-34b and miR-34c expression, and associated to a down-stream increase of c-Myc protein level (**Supplementary Fig. 7b**).

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. 8). We next set out to demonstrate that altered Mdm2 protein expression is responsible for the USP2a-driven regulation of miR34b/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 over-expression of miR-34b/c (Fig. 3c). These results are supported by ChIP results, clearly demonstrating a reduced p53 binding to the RE2 sequence (as seen with USP2aWT over-expression) in cells over-expressing Mdm2 (Fig. 3d). Conversely, siMdm2 in the USP2aWT clone activates p53, and enhances its down-stream recruitment to both the RE1 and RE2 containing sequences (Fig. 3d). Furthermore, treatment with Nutlin-3a (a specific Mdm2 inhibitor) leads to a dose-dependent activation of p53 in LNCaP cells, with a resultant up-regulation of miR-34b/c and down-regulation of c-Myc (Fig. 3e). Different p53 putative binding boxes have also been identified along the 5’ upstream regions (up to 50 kb) of both miR-98 and let-7c genomic loci (Supplementary Fig. 9a). Both miRs are modulated by USP2aWT and are directly involved in the USP2a-mediated modulation of c-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. 9b) together with siUSP2a and Nutlin-mediated p53 induction (Supplementary Fig. 9c) are both unable to affect miR-98/let-7c expression, and unable to trigger p53 recruitment to its response elements in miR regulatory regions in LNCaP cells (Supplementary Fig. 9d). Our data suggest that in prostate cancer cells, USP2a-mediated induction of Mdm2, and degradation of p53, impedes the transcription of the miRNA cluster miR-34b/c.
To verify the biochemical effect of USP2a expression on the Mdm2-p53 pathway and subsequent impact on c-Myc protein expression, we carried out a time-course experiment and measured the proteins levels of USP2a, Mdm-2, p53 and c-Myc and miR34b/c at different set points following USP2aWT over-expression in LNCaP cells (Fig. 4). USP2a expression started at 12 hours post-transfection and is sustained, during the first 36 hours. Subsequently, Mdm2 is up regulated (24-36h) and p53 down regulated (36-60h). While the decrease in miR34b/c expression occurs as early as 12 hours following USP2a transfection, as an overall effect, c-Myc expression is up-regulated by 60-72h. We confirmed this results employing a prostate cell line (PC3) devoid of p53. When we restore wild type p53 presence under USP2a expression, we observe an up-regulation of c-Myc, as observed in LNCaP cells. Conversely, non-degradable p53 mutants (Δ13-19 or 22Gln/23Ser) and USP2a co-expression led to a decrease in c-Myc expression (data not shown). Taken together, these results suggest that USP2a regulates c-Myc expression via the Mdm2-p53 axis.

mRNA signature of USP2a over-expression

As validation, we next analyzed differential gene expression in iPrEC transfected with USP2aWT and USP2aMUT using the Affymetrix Human Genome U133 Plus 2.0 Array (Fig. 5). Empty vector was used as 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 USP2aWT. 789 genes were differentially expressed between USP2aWT and USP2aMUT; 848 genes were differentially expressed between
USP2a\textsuperscript{WT} and EV; 54 genes were differentially expressed between USP2a\textsuperscript{MUT} and EV (p-value < 0.0001 after adjustment for multiple testing in all pair-wise comparisons). USP2a over-expression was associated with a global up-regulation in mRNA transcripts and several cancer-related genes such as c-\textit{myc} were highly expressed in the USP2a\textsuperscript{WT} group compared to both USP2a\textsuperscript{MUT} and EV cell lines. Complete lists of differentially expressed genes for pair-wise comparisons can be accessed online\textsuperscript{31}. We further show that the gene expression signature of USP2a\textsuperscript{MUT} and EV cell lines is indistinguishable from each other, and differs markedly from the discrete gene expression signature associated with USP2a\textsuperscript{WT}, both at the gene and pathway levels (Fig. 5a; see also volcano and "concordance-at-the-top" plots shown in Supplementary Fig. 10 and Supplementary Fig. 11).

We next applied the one-sided Wilcoxon rank-sum test to investigate differential enrichment of 86170 Functional Gene Sets (FGS) corresponding to specific annotation and biological themes in iPrEC transfected with USP2a\textsuperscript{WT} and USP2a\textsuperscript{MUT} relative to EV control. FGS were retrieved from a variety of genomics databases, enabling the interrogation of cellular pathways, protein networks, transcription factors, miRNA targets, genomic locations, enzymatic activities and cellular processes. This analysis highlighted c-Myc-related biological pathways associated with USP2a\textsuperscript{WT} over-expression (in contrast to USP2a\textsuperscript{MUT} and EV). Global up-regulation of genes transcribed by c-Myc\textsuperscript{32} and miR-34c expression\textsuperscript{33} was mirrored by the overall down-regulation of genes repressed by p21 in a p53-dependant manner\textsuperscript{34} and targeted by miR-19a\textsuperscript{33} (Fig. 5b). The complete thematic list and functional pathways differentially enriched between USP2a\textsuperscript{WT} and USP2a\textsuperscript{MUT} cell lines can be accessed online\textsuperscript{31}. Finally, we compared both
USP2a\textsuperscript{WT} and USP2a\textsuperscript{MUT} specific gene expression signatures (i.e. the non-overlapping segments of the Venn diagrams in Fig. 5a) to a large laser capture micro-dissected (LCM) prostate cancer data set available from the public domain (GSE69099 data set, ref. 35, Fig. 5c). The Wilcoxon rank-sum test was used to evaluate whether up- and down-regulated genes were consistently enriched along Prostate Cancer (PCA) progression. Genes up-regulated upon transfection of USP2a\textsuperscript{WT} are enriched in the epithelial compartment of human prostate cancer while genes up-regulated by USP2a\textsuperscript{MUT} are enriched in the stromal compartment. Interestingly, USP2a\textsuperscript{WT} genes appeared to be strongly up-regulated during local invasion of cancer cells, since they were most strongly enriched when locally invasive cancers were compared to Prostatic Intraepithelial Neoplasia (PIN) specimens (Fig. 5c).

**Targeting c-Myc in USP2a over-expressing cells suppresses growth and invasion**

USP2a modulation of c-Myc protein expression may have important biological implications for the natural history of cancer. We demonstrate that the proliferative capacity, cell viability and clonogenic ability of LNCaP USP2a\textsuperscript{WT} cells can be significantly impaired by targeting c-Myc expression. This can be achieved by direct silencing of c-Myc, by administration of Nutlin-3a or by treating USP2a\textsuperscript{WT} cells with synthetic miR 34b/c molecules (Supplementary Fig. 12). Since the genes up-regulated by USP2A\textsuperscript{WT} proved to be enriched during local invasion of prostate cancer (Fig. 5c), we next profiled the mRNA signature of a number of distinct cancer invasion models. CAT-plot analysis revealed significant
agreement between genes up-regulated by USP2a\textsuperscript{WT} and those up-regulated upon 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, while overlap was observed when the comparison involved the “round/branching” non-invasive phenotypes (data not shown). The “stellate” phenotype corresponds to invading cancer cell lines, the “mass” phenotype to non-invasive cancer cell lines and transformed normal prostatic epithelial cells, and the “round/branching” one to PrECs and non-transformed lines. In agreement with the CAT-plot analysis, the in vitro invasion test demonstrates that UPS2aWT over-expression increases the invasive ability of iPrEC when compared to control or USP2aMUT-transfected cells, and, when silenced in an LNCaP experimental model, reduces invasion by almost 2-fold (Fig. 6c). miR-34b/c seem to be involved in the USP2a-mediated invasiveness, as modulation of their expression by either mimic treatment in iPrEC\textsubscript{USP2aWT} cells or by antago-miR addition to USP2a-silenced LNCaP cells is able to significantly affect the relative prostate invasive ability (Fig. 6c).
DISCUSSION

Deubiquitinating enzymes can prevent destruction of protein substrates prior to proteosomal degradation. USP2a deubiquitinates the anti-apoptotic proteins Fatty Acid Synthase (FAS), the ubiquitin ligase Mdm2 and Cyclin D1. USP2a is overexpressed in ~40% of human prostate adenocarcinomas\textsuperscript{24}, and exhibits oncogenic behavior both \textit{in vitro} and \textit{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 c-Myc\textsuperscript{1,36-39}. Importantly, this study highlights a previously unrecognized mechanism of c-Myc regulation establishing a novel link between de-ubiquitination, miRNA deregulation and the p53/c-Myc regulatory network. Gene set enrichment analysis of microarray expression in human tumors highlights a c-Myc related gene set supporting the link between USP2a and c-Myc. Indeed, the c-Myc related gene set associated with USP2a\textsuperscript{WT} over-expression is differentially expressed between cancer and normal prostate and, importantly, these genes are associated with prostate tumor progression. Specifically, we show that the proliferative capacity, cell viability, clonogenic ability and invasive properties of USP2a\textsuperscript{WT} over-expressing cells can be significantly impaired by targeting c-Myc expression. Although novel therapeutics to target Myc-dependent tumors have been developed\textsuperscript{40-42} these are
generally not effective. This study provides evidence that, at least in prostate cancer, myc may be affected by targeting either USP2a or Mdm2.

Although studies addressing the role of miRNAs in cancer pathogenesis are at an early stage, it is clear that miRNA expression patterns correlate strikingly with disease progression, and response to therapy\(^4^3\). However, the biological roles of only a very limited number of miRNAs have been elucidated in cancer cells. The down-regulation 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 USP2a\(^W^T\) is the first example of ubiquitin-proteasome pathway playing a role in miRNA biology. We outline our proposed model for this mechanism in Fig. 7.

Several studies have demonstrated the utility of developing specific inhibitors of ubiquitin ligases and deubiquitinating enzymes as valuable therapeutic strategies against cancer\(^4^4,4^5\). Our results further underscore the importance of these therapeutic strategies. Analogously, USP2a inhibitors may offer a potentially viable means of targeting Myc-dependent prostate cancer.

**METHODS**

**Cell culture.** Empty vector (Vector), wild type (USP2a\(^W^T\)) and mutant (USP2a\(^M^U^T\)) stable clones (previously established by infecting immortalized androgen receptor (AR)-expressing prostate epithelial cells (iPrEC)\(^2^3,2^4\) were grown in specific PrEBM medium (Cambrex, East Rutherford, NJ, USA) and selected in 1.6 \(\mu\)g/ml puromycin. Human prostate adenocarcinoma LNCaP and PC3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and
grown in RPMI-1640 medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) 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 three months) for cell morphology, growth rate, colony forming ability and gene expression of both p53 and AR. Moreover, cells have undergone 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 (2x10^5 cells/plate). Transfection was performed 24 hours after plating using Lipofectamine 2000 reagent in Optimem medium (Invitrogen) for siRNA oligonucleotides (100-500 nM) and synthetic/knockdown miR molecules (10-50 nM), and by JetPei reagent (PloyPlus-Transfection, New York, NY, USA) 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 luciferase reporter assays, cells were co-transfected with the pGL3-c-Myc 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^WT^ cells. 48 to 96 hours later, cells were rinsed with cold PBS, re-suspended in cell lysis buffer (Promega, WI, USA), and incubated for 10 min at room temperature. Insoluble material was spun down,
and luciferase activity was quantified by a commercially available kit (Promega) on a TD-20E luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luciferase values were normalized to protein contents and β-galactosidase expression. All sequences of siRNA are reported in Supplementary Table 1.

**Western blot.** Cell lysis was performed on ice for 30 min 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 PMSF, 1 μg/ml aprotinin, leupeptin, pepstatin). Equal amounts of total protein extracts (20-80 μg) were resolved by 10%, 12% or 15% denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred for 4 hours to polyvinylidene difluoride membrane. Membranes were blocked in 5% milk-PBS-0.05% Tween 20 for 1 hour and incubated overnight with the specific primary antibodies. Secondary antibodies were horseradish peroxidase-conjugated (Santa Cruz, Santa Cruz-CA, USA), and ECL reagent (Amersham, GE Healthcare, Piscataway, NJ, USA) was employed for chemo-luminescence detection.

**RNA isolation and qPCR for mRNA analysis.** Total RNA was extracted using Trizol Reagent (Invitrogen). RNA quantity and integrity was assessed using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), 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. Semi-quantitative RT-
PCR was performed with the Applied Biosystem Taq enzyme (Applied Biosystems by Life Technologies, Foster city, CA, USA), using 2 μl of cDNA. PCR conditions were as follows: 1 cycle at 94°C (5 min); 23-35 cycles at 94°C (30 s), 58°C (40 s), 72°C (40 s); 1 cycle at 72°C (7 min). PCR products were run on a 2% agarose gel and visualized with ethidium bromide. Gene transcript expression was normalized to GAPDH levels. Quantitative RT-PCR (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 GAPDH and Aldolase A expression using the $2^{-\Delta\Delta CT}$ method. All sequences of oligonucleotide primers are reported in Supplementary Tables 2-3.

**Immunoprecipitation of miRNA targets.** PC3 cells (0.5 x 10⁶ cells) were co-transfected with 1μg of HA-AGO1 (pIRES-FLAG/HA vector, Addgene, Cambridge, MA, USA) and 50 nM of miRNA mimics using Lipofectamine 2000 (Invitrogen). After 2 days cells were harvested using 400 μl lyses 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, Indianapolis, USA). 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 h at 40 C in Spin Columns-Screw Cap (Pierce, Rockford, IL, USA). The columns were drained, washed and the retained beads were treated with 5 U DNases in NT2 buffer (50 mM Tris, pH7.4,
150 mM NaCl, 1 mM MgCl2, 0.05 % Nonidet P-40) for 10 min at 37°C, washed with NT2 and then treated with Proteinase K in NT2 plus 1% SDS for 30 min at 55°C. Finally the beads were re-suspended in NT2 buffer and RNA was extracted using acid phenol-chloroform (Ambion-Applied Biosystems) to extract RNA. RNA was reverse transcribed and analyzed with qPCR for enrichment of c-myc mRNA.

**Chromatin Immunoprecipitation (ChIP) assay.** Cells were washed with PBS (supplemented with phosphatase inhibitors) and incubated for 10 min with 1% formaldehyde at room temperature. Formaldehyde cross-linking was stopped by adding glycine, pH 2.5 (125 mM final concentration), for 5 min at room temperature. Cells were scraped off the plates, lysated in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, plus protease inhibitor mixture), and sonicated to generate 500-2000bp 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 (BSA)). The cleared lysate was incubated over-night 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% SDS and 100 mM sodium carbonate. DNA-protein cross-links were reversed by heating at 65 °C for 4-5 h, and DNA was phenol-extracted and ethanol-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. PCR conditions were as follows: 1 cycle
at 94°C (5 min); 28-35 cycles at 94°C (30 s), 58°C (40 s), 72°C (40 s); 1 cycle at 72°C (5 min). 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 4-7.

**qPCR for miRNA analysis.** Analysis of miRNA expression was carried out on total RNA using real-time stem-loop RT-PCR according to the manufacturer’s protocol (Taqman miRNA assays are available as assays on demand (Applied Biosystems). Briefly, 10ng of total RNA was reverse-transcribed with specific miR stem-loop primer, and subsequently amplified by miR specific primers. Q-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 nucleolar RNAs (RNU) using the $2^{- \Delta \Delta Ct}$ method$^{46}$.

**In vitro cell Invasion analysis.** Cell invasion ability was assessed using the Chemicon Invasion Chamber (Chemicon, Millipore, Billerica, MA, USA), 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% FBS for iPrEC and LNCaP cells, respectively) were added to the lower chamber, while 300 μl of cell suspension (10⁶ cells/ml) of
iPrEC clones in PrEBM and LNCaP cells in RPMI plus 2% FBS (siControl or siUSP2a-treated prior to 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, non-invading cells were gently removed from the interior of the inserts with a cotton-tipped swab. Invasive cells on 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 is presented as mean ± sd calculated ≥ 3 replicates. Statistical analyses were performed using an unpaired two-tailed t test using SPSS Software Version One. 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 I (Qiagen, Valencia, CA, USA) digestion step and further enriched using Qiagen RNeasy columns. RNA quantity and integrity was assessed using a NanoDrop Spectrophotometer (Thermo Scientific), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively. Five replicates of EV, USP2a^WT, USP2a^MUT respectively were run on the Human Genome U133 Plus 2.0 Array from Affymetrix as previously described. A detailed description of the methods used for array data annotation, processing and analysis and public data sets used in the present study are described in the Supplementary Methods section.
Analysis of mRNA signature of distinct cancer invasion models. (a) Migration efficiency/velocity in Boyden assays: upon a review of the literature (see supplementary references) we identified a number of cell lines from various cancer types displaying extreme differences in migration efficiency/velocity as assayed in Boyden assays, as reported in Supplementary Tables 8-10, and retrieved the corresponding Affymetrix raw gene expression data from the NCBI GEO database. Using the same generalized linear model approach applied to analyze gene expression upon USP2a transfection, we analyzed differential gene expression between fast and slow migrating prostate cancer cell lines (PC3 and LNCAP respectively) (Supplementary Table 9). Similarly, in order to avoid confounding with experimental batch and cell lines characteristics (i.e. androgen sensitivity), we also analyzed migration efficiency/velocity in Boyden assays in a balanced compounded dataset accounting for several cancer cell lines of different tissue origin) (Supplementary Table 10). (b) Three-dimensional model of prostate cancer invasion: we also analyzed differential gene expression associated with prostaspheres displaying distinct patterns of invasion in Matrigel. To this end we retrieved and analyzed gene expression data from the GSE19426 series (1), comparing prostaspheres with distinct growth and invasion behavior (“round”, “branching”, “mass”, and “stellate”, as described by Harma and colleagues (see supplementary references), using a generalized linear model approach as applied to analyze gene expression upon USP2a transfection. A description of the phenotypic groups compared is available in Supplementary Table 11. (c) Prostate gland branching morphogenesis: the gene expression signature of prostate gland branching morphogenesis was obtained by comparing mouse male and female
urogenital sinuses at gestational day 17 (+- 0.5), as analyzed in Schaeffer and colleagues (see supplementary references) (GSE12077).

ACKNOWLEDGMENTS

The authors would like to thank Carmen Priolo for providing iPrEC USP2a\textsuperscript{WT} and USP2a\textsuperscript{MUT} cells, Prof. Moshe Oren (Weizmann Institute of Science, Rehovot, Israel) for kindly providing the pCDNA3-Mdm2 (pMdm2) expression vector, and Dr. Yatrik Shah (National Cancer Institute, Bethesda, MD, USA) for sending us the pGL3-LUC-Myc-3'-UTR. The authors would also like to thank Stefano Sioletic for helpful contribution to the assessment of USP2a-myc relationship in human specimens.
REFERENCES


48. http://genome.ucsc.edu/cgi-bin/hgGateway
FIGURE LEGENDS

**Figure 1** USP2a over-expression modifies the microRNA expression profile of prostate cells. (a) Analysis of USP2a mRNA and protein expression in empty vector (Vector), USP2a\textsuperscript{MUT} and USP2a\textsuperscript{WT} iPrEC transfectants. (b) Prostate-specific miRNA expression profiling color heat-map carried out by TaqMan qRT-PCR assay in the indicated iPrEC cells. Evaluation of USP2a protein expression and miRNA level analysis performed in LNCaP cells upon: (c) transfection with empty vehicle (Ve), pCDNA3 (Vector), pUSP2a\textsuperscript{MUT} or pUSP2a\textsuperscript{WT}; and (d) incubation with empty vehicle (Ve), control (siControl) or specific USP2a (siUSP2a) silencing oligonucleotides. All data represent mean ± s.d. of at least three independent replicates. $P$ values: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. See also Supplementary Fig. 1.

**Figure 2** USP2a-dependent miRs deregulation modulates c-Myc expression. (a) Western blot analysis of c-Myc protein level, and evaluation of c-Myc specific transcription target genes carried out in USP2a over-expressing iPrEC cells. (b) Evaluation of c-Myc protein level (left panel), c-Myc-regulated transcripts and miRNAs (right panels) performed in empty vector iPrEC cells (Vector) and iPrEC\_USP2a\textsuperscript{WT} cells silenced for either GFP or Myc expression. Analysis of miR-34b and miR-34c (c), and c-Myc expression levels (d) carried out in iPrEC\_USP2a\textsuperscript{WT} cells following treatment with increasing concentrations of control (CTR), miR-34b and miR-34c synthetic molecules. (e) Luciferase (LUC) reporter assay performed in empty vector (Vector), USP2a\textsuperscript{MUT} and USP2a\textsuperscript{WT} iPrEC transfectants (top panel), and in USP2a\textsuperscript{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 co-transfected with expression vectors for HA-tagged AGO1 and the indicated miRNA 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 served as controls, which are known to target c-Myc. Relative to input c-Myc was significantly (p<0.002) enriched in the pull-down with miR-34b and miR-34c. All data represent mean ± s.d. of at least three independent replicates. \( P \) values: (*) \( P < 0.05 \), (**) \( P < 0.01 \), (***) \( P < 0.001 \). See also Supplementary Fig. 2-5.

**Fig. 3.** (a) Schematic representation of the genomic region mapping 4Kb up-stream of the miR-34b/c cluster site on Chromosome 11 (position 11q23.1: [110,888,873-110,889,450]). DNA fragments amplified by each oligonucleotide pair are represented by arrows (A-O). (b) ChIP assay of LNCaP cells with immunoprecipitation of chromatin without antibody (no Ab) and with specific anti-p53, p300, AcH4, phosphorylated Pol-II or control IgG antibodies. PCR amplification of amplicons A-O on the putative miR34b/c regulating region, on the p21 promoter (positive control) and on the cyclin B1 intron (negative control) are highlighted. (c) Western blot evaluation of Mdm2 (top panel) and miRNA 34b/c expression analysis (bottom panel) carried out in LNCaP cells, following transient transfection with empty vector (Vector), pMdm2 expression vector, and in USP2aWT_LNCaP transfectants silenced with either control (siGFP) or specific Mdm2 siRNAs.
(d) p53-ChIP assay (as described in (b), top panels) and evaluation of c-Myc protein levels (bottom blots), performed in LNCaP cells, following transient transfection with empty vector (Vector), pMdm2 expression vector, and in USP2aWT_LNCaP transfectants silenced with either control (siGFP) or specific Mdm2 siRNAs. (e) Western blot of p53 and c-Myc protein levels (top panel) and miR-34b and miR-34c expression levels (bottom panel) carried out in LNCaP cells treated with increasing concentrations of Nutlin-3a in DMSO. All histograms represent mean ± s.d. of at least three independent replicates. P values: (*) P< 0.05, (**) P< 0.01, (***) P< 0.001

**Figure 4** USP2a regulates c-Myc expression via the Mdm2-p53 axis. (A) Kinetics profile (0-72 h) of protein levels in LNCaP cells following pUSP2a-WT (black bars) over-expression or empty vector (white bars). Representative blots are shown at each set point for the specific protein (upper panel) and actin levels (lower panel). (B) miR34b/c expression profile under the same conditions depicted in (A).

**Figure 5** USP2a over-expression is associated with a discrete mRNA signature which is enriched for c-Myc regulated transcripts. (a) Venn diagram of differentially expressed genes in iPrEC transfected with USP2a^WT^ and USP2a^MUT^ and Empty vector (EV) control. Nominal adjusted P-value < 0.0001; results are for all non-redundant NCBI ENTREZ Gene identifiers available on Affymetrix U133 Plus 2.0 Array. Red = up-regulated genes; green = down-regulated genes. (b) Functional Annotation Analysis for genes differentially expressed between USP2a^WT^ and USP2a^MUT^. Genes were ordered by moderated t-statistics obtained from USP2a^WT^
and USP2a\textsuperscript{MUT} contrast, while Functional Gene Sets (FGS) where obtained from different data bases (see Supplementary Microarray Methods and Results sections). K5Z233_V_MYCMAX_01 and K5Z233_V_NMYC_01: genes that contain a Transcription Factor Binding Site (TFBS) for the heterodimer Myc-Max or n-Myc in the 10Kb genomic region around their transcription starting site (TSS) with a False Discovery Rate (FDR) <1\textsuperscript{48}; UNION_hsa_miR_34c and UNION_hsa_miR_19a: union sets of genes described as targets for miR-34c or miR19a by DIANA-microT, miRanda, TargetScanS, and PicTar, as obtained from the miRGen database\textsuperscript{33}; MYC_ONCOGENIC_SIGNATURE: cMYC target genes\textsuperscript{32}, FGS obtained from the Molecular Signature Database (MSigDB); P21_P53_ANY_DN: p21 down-regulated (p53-dependent) genes\textsuperscript{32}, FGS from the Molecular Signature Database (MSigDB); YU_CMYC_UP: c-Myc target genes in a p53-null background, FGS from the MSigDB. Analysis revealed enrichment by up-regulation for Myc and miR-34c target genes, and enrichment by down-regulation for p21-p53, miR-19a target genes. Color values represent absolute log\textsubscript{10} adjusted \(P\)-values resulting from the Wilcoxon rank-sum test, after multiple testing correction. Orange/red boxes show concordant up-regulation, while blue boxes show concordant down-regulation. (c) Enrichment analysis of genes up- and down-regulated between USP2a\textsuperscript{WT} and USP2a\textsuperscript{MUT} in sequential transitions along human prostate cancer progression, as analyzed in the GSE6099 data set\textsuperscript{35}. The following transitions were considered: Neoplasia: PIN epithelial cells \textit{versus} NORMAL epithelial cells; Cancer: PIN and PCA epithelial cells \textit{versus} NORMAL epithelial cells; Invasion: PCA epithelial cells \textit{versus} PIN epithelial cells; Gleason: High Gleason (<7) PCA epithelial cells \textit{versus} Low Gleason (>7) PCA epithelial
cells; Progression: Metastatic PCA epithelial cells versus locally invasive PCA epithelial cells; AR independence: Androgen-resistant metastatic PCA epithelial cells versus androgen-naive metastatic PCA epithelial cells; Epi versus Stro: LCM epithelial cells versus LCM stromal cells; Stro PCA versus Stro NOR: LCM from stromal cells adjacent to PCA versus LCM stromal cells from normal prostate. Color values represent absolute log_{10} P-values resulting from the Wilcoxon rank-sum test. Orange/red boxes show concordant up-regulation, while blue boxes show concordant down-regulation. See also Supplementary Fig. 9-10.

**Figure 6** USP2a over-expression increases the invasive ability of prostate cells. Concordance At the Top plot (CAT-plot) showing the agreement between USP2a and invasion gene expression signatures. Genes up-regulated by wild type USP2a transfection as compared to mutant USP2a showed significant overlap with genes expressed upon invasion in a number of distinct laboratory models. On the y-axis is shown the proportion of genes in common, on the x-axis the number of top ranking genes considered. The black line corresponds to proportions of common genes by chance, grey 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 pair-wise comparison between differential gene expression between wild type and mutant USP2a compared to 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 cell lines in Boyden assays (blue line); C) mouse male and female urogenital sinuses at gestational day 17 (+- 0.5) (green line); D) Matrigel invasive and non invasive prostaspheres (purple line); E) LCM epithelial cells from locally invasive prostate cancer and 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 (+- 0.5) (green line); D) Matrigel invasive and non invasive 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), while USP2a-silenced LNCaP cells underwent further treatment with specific antagomiRs (KO molecules), before invasion assessment. Ve stands for the empty vehicle used for molecule administration. Data represent mean ± s.d. of four independent replicates. P values: (*) P< 0.05, (**) P< 0.01.

**Figure 7** Proposed model of how USP2a-driven miRNAs deregulation enhances c-Myc protein expression through impairment of the Mdm2-p53 pathway. p53 regulates miR-34b/c expression through promoter enhancement. (A) When over-expressed, USP2a triggers the down-regulation of miR-34b/c with subsequent c-Myc activation via Mdm2-mediated p53 inactivation. (B) When USP2a is under-
expressed, it triggers the up-regulation of miR-34b/c with subsequent c-Myc deactivation via Mdm2-mediated p53 activation.
Figure 1

(a) iPrEC

(b) iPrEC

(c) LNCaP

(d) LNCaP

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Figure 2

(a) iPrEC

- c-Myc
- β-actin
- cycl B1
- cdc25a
- cdk4
- odc
- darc
- gapdh

Vector
USP2aWT
USP2aAT

(b) iPrEC_USP2aWT

- c-Myc
- tubulin

siGFP
siMyc
siMyc

48h
72h

(c) iPrEC_USP2aWT

Average of ΔΔCt vs. RNU24, RNU44, RNU48

- miR-17-5p
- miR-18a
- miR-19a
- miR-20a
- miR-92-1

(d) pGL3_LUC-myc-3'UTR

Relative LUC activity

- Vector
- USP2aWT
- USP2aAT

(e) pGL3_LUC-myc-3'UTR

Relative LUC activity

- CTR
- 34b
- 34c

(f) SS rRNA
- c-Myc

miR-98
let-7c
miR-34c
miR-34b

Fold Enrichment

0
5
10
15
20

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Figure 3

a

Chr 11q23.1[110886873-110869450].miR-34b/c cluster

b

![DNA fragment analysis](image)

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Figure 4

(a) Relative protein expression vs. actin for USP2a and Mdm2.

(b) Fold difference for miR-34b and miR-34c at 0h, 12h, and 72h.
Figure 5

(a) Differentially expressed genes (adjusted P-value < 0.0001)

(b) Heatmap of gene expression values

(c) Additional gene expression data
Figure 6

(a) Decreasing relative stats: USP2A vs Invasion
(with PCA vs PIN)

(b) Decreasing relative stats: USP2A vs Invasion
(without PCA vs PIN)

(C) iPrEC

Relative invasive ability

Vector USP2aMUT USP2aWT

LNCaP

Relative invasive ability

Vector siControl siUSP2

iPrEC_USP2aWT

Vector Mimic CTR Mimic 34b Mimic 34c

LNCaP

relative invasive ability

Vector KO_scr KO_34b KO_34c
Figure 7

(a) USP2a and Mdm2 promote miR-34b and miR-34c, leading to an increase in C-Myc.

(b) p53 and Mdm2 negatively regulate miR-34b and miR-34c, inhibiting C-Myc expression.
c-Myc is activated via USP2a-mediated modulation of microRNAs in prostate cancer

Barbara Benassi, Richard Flavin, Luigi Marchionni, et al.

Cancer Discovery  Published OnlineFirst January 5, 2012.

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