PPP2R2C Loss Promotes Castration-Resistance and Is Associated with Increased Prostate Cancer-Specific Mortality

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Abstract
Metastatic prostate cancers generally rely on androgen receptor (AR) signaling for growth and survival, even following systemic androgen-deprivation therapy (ADT). However, recent evidence suggests that some advanced prostate cancers escape ADT by using signaling programs and growth factors that bypass canonical AR ligand-mediated mechanisms. We used an in vitro high-throughput RNA interference (RNAi) screen to identify pathways in androgen-dependent prostate cancer cell lines whose loss-of-function promotes androgen ligand-independent growth. We identified 40 genes where knockdown promoted proliferation of both LNCaP and VCaP prostate cancer cells in the absence of androgen. Of these, 14 were downregulated in primary and metastatic prostate cancer, including two subunits of the protein phosphatase 2 (PP2A) holoenzyme complex: PPP2R1A, a structural subunit with known tumor-suppressor properties in several tumor types; and PPP2R2C, a PP2A substrate-binding regulatory subunit that has not been previously identified as a tumor suppressor. We show that loss of PPP2R2C promotes androgen ligand depletion-resistant prostate cancer growth without altering AR expression or canonical AR-regulated gene expression. Furthermore, cell proliferation induced by PPP2R2C loss was not inhibited by the AR antagonist MDV3100, indicating that PPP2R2C loss may promote growth independently of known AR-mediated transcriptional programs. Immunohistochemical analysis of PPP2R2C protein levels in primary prostate tumors determined that low PPP2R2C expression significantly associated with an increased likelihood of cancer recurrence and cancer-specific mortality. These findings provide insights into mechanisms by which prostate cancers resist AR-pathway suppression and support inhibiting PPP2R2C complexes or the growth pathway(s) activated by PPP2R2C as a therapeutic strategy.

Introduction
Prostate cancer is the second leading cause of cancer-related mortality in American men (1). Most deaths are attributable to metastatic disease, a stage that exhibits high initial response rates to systemic androgen-deprivation therapy (ADT; ref. 2). Unfortunately, nearly all metastatic tumors recur as castration-resistant prostate cancers (CRPC; ref. 2). Currently available treatments of CRPC provide limited improvements in survival, though recent developments of novel immunotherapies and more potent antiandrogens have resulted in additional modest increases in patient longevity (3–5).

Importantly, the vast majority of CRPCs reactivate androgen receptor (AR)-mediated transcriptional networks that seem to be necessary for prostate cancer survival and growth. The maintenance of AR-pathway activity has been shown to occur through a variety of mechanisms, including but not limited to transcriptional and genomic amplification of AR, mutations of AR that allow promiscuous ligand binding, generation of constitutively active AR splice variants, de novo synthesis of intratumoral androgens, and transactivation of the AR by intracellular signal transduction programs (6, 7). However, there is mounting evidence that alternative mechanisms can promote CRPC progression independent of AR activation. Several recent in vitro and in vivo studies provide evidence that phosphoinositide 3-kinase (PI3K) pathway signaling is sufficient for CRPC survival in the setting of low or absent AR activity (8, 9). With clinical efforts focused on extinguishing AR activity through enhanced AR blockade and the elimination of AR
ligands, additional androgen pathway-independent resistance mechanisms are likely to emerge.

To identify genes and pathways that modify prostate cancer growth in the context of suppressed AR signaling, we conducted in vitro high-throughput RNA interference (RNAi) screening using a siRNA library designed to target 6,650 individual genes representing several major oncology classes including cellular kinases, phosphatases, transcription factors, and growth factor receptors. We hypothesized that a subset of genes and gene networks could confer a castration-resistant phenotype in prostate cancer cells previously dependent upon androgen-mediated signaling for growth and survival. To prioritize candidate hits for clinical relevance, the HTRS results were cross-referenced with gene expression datasets collected from primary prostate cancers and cases of metastatic CRPC. This evaluation revealed that a subset of transcripts encoding proteins comprising the protein phosphatase 2A (PP2A) complex were enriched for a phenotype of AR ligand-independent growth promotion in the HTRS experiments and were also downregulated in a subset of primary and metastatic prostate carcinomas.

PP2A is a highly conserved serine/threonine phosphatase that has a broad spectrum of biologic roles including the negative regulation of signal transduction, cell-cycle progression, and gene expression (10, 11). The PP2A holoenzyme is composed of a core dimer—consisting of a catalytic subunit (PPP2CA/PPP2CB) and a structural subunit (PPP2R1A/PPP2R1B)—which forms a heterotrimERIC complex with a “B” subunit. The B subunit originates from one of 18 genes grouped into four structurally unrelated families and is thought to dictate substrate specificity, cellular localization, and enzymatic activity of the PP2A complex (12). Loss of PP2A activity is often a transforming event, which is perhaps most obvious in cells expressing the SV40 small t antigen (SV40ST) viral oncogene. SV40ST competes with B subunits to bind the PPP2C heterodimer (13); SV40ST binding is required for viral transformation (14). In addition, nonviral mechanisms of PP2A inhibition are well documented in a wide variety of human cancers, and de novo inactivating mutations have been identified in each subunit class of the PP2A holoenzyme (10).

In prostate cancer, decreased expression of the catalytic subunit PPP2CA and the regulatory subunit PPP2R2A have been observed in a subset of primary tumors (15, 16). Studies of PP2A in ADT-resistant prostate cancers determined that loss of PPP2CA function is sufficient to confer AR ligand-depleted growth in vitro through a mechanism that sustained AR transcriptional activity (17). In the present report, we identified 4 PP2A components that, when downregulated, induce ADT-resistant prostate cancer cell growth. Of these, transcripts encoding the PP2A A- and B-regulatory family subunits, PPP2R1A and PPP2R2C, respectively, were significantly decreased in a subset of ADT-resistant primary and metastatic prostate cancers. Loss of PPP2R2C associated with increased prostate cancer metastasis and cancer-specific mortality. Importantly, we determined that loss of PPP2R2C promoted prostate cancer cell growth in the absence of AR ligands without upregulating AR expression or activating AR target genes. The ADT-independent growth conferred by PPP2R2C suppression did not occur via activation of cSRC, P13K, or extracellular signal-regulated kinase 1/2 (ERK1/2) signaling. Collectively, these studies provide insights into mechanisms capable of conferring androgen-independent prostate cancer progression that does not rely on AR activation and suggest that strategies capable of promoting PP2A activity, or modulating signaling programs regulated via PP2A, should be evaluated for therapeutic effects in patients with CRPCs.

Materials and Methods

Cell culture and siRNA transfection

LNCaP and VCaP cell lines were obtained from American Type Culture Collection and maintained in androgen-replete phenol red-free RPMI-1640 + 10% FBS (Life Technologies) + 1% PenStrep (Life Technologies). Cells were used within 6 months of resuscitation. One day before transfection cells were passaged into androgen-deplete phenol red-free OptiMEM (Life Technologies) supplemented with 3% charcoal-dextran–stripped FBS (CSS) onto Matrix Screen Mate tissue culture-treated 384-well plates (Thermo Scientific) using the densities determined during the feasibility study on a FluidX XRD384 peristaltic liquid handler in a total well volume of 50 μL.

Experimental working plates were prepared by first stamping 10 μmol/L siRNA library stocks into dry 384-well Abgene 1055–384-well microarray plates (Thermo Scientific) using a Vario workstation (Cybio) equipped with a 25 μL 384-well tip head. A sidecar plate containing UNI1 nontargeting control, Kif11, and assay-specific controls (Sigma-Aldrich) was then replicated on each experimental working plate on the Cybio Vario workstation. OptiMEM and Lipofectamine RNAiMax (Life Technologies) were added to the experimental working plates on a FluidXXRD384 peristaltic liquid handler using predefined metrics determined via the Quellos HTS Feasibility assay. Transfections were carried out by adding 5 μL for VCAP and 0.625 μL for LNCAP, respectively, from each experimental working plate onto 3 replicate cell plates using the CyBio Vario workstation.

Cell viability was assessed after the 96-hour incubation with the addition of Cell Titer-Glo (Promega) on the FluidX 384 liquid handler. Luminescence was read using an Envision HTS multilabel reader (PerkinElmer).

Deconvoluted siRNA experiments were carried out in 96-well plates using transfection conditions scaled-up from 384-well experiments in a total volume of 125 μL. Kif11, UNI1 nontargeting control, and PPP2R2C-targeting siRNA were purchased from Sigma-Aldrich. Ninety-six hours after transfection, cell viability was assessed by adding Cell Titer-Glo and measuring luminescence on a Synergy2 multiwell plate reader (BioTek). siRNA sequences can be found in Supplementary Table S2.

Dasatinib was purchased from Chemitek, R1881 was purchased from PerkinElmer, U0126 was purchased from Cell Signaling Technology, and LY294002 was purchased...
from Promega. MDV3100 was obtained as a gift from Medivation Inc.

**Statistical analysis of siRNA experiments**

The data from the HTRS experiments were defined as the raw signal luminescence measured by the Envision HTS multilabel reader. For each sample, the raw intensities were transformed to Z scores within each plate. The median standardized value for each gene was then used as an input for the downstream analysis. To downweight the influence of siRNAs undergoing large changes on the parameters used to define the Z score transformation, the estimated means and variances were defined after removing the top and bottom 2.5% of the data within each plate.

Analysis of follow-up siRNA screens and quantitative real-time PCR (qRT-PCR) data was conducted in Prism 4.0 software (GraphPad). Raw luminescence was normalized to the mean luminescence value of siUNI for each cell line. Treatment effect was measured by normalizing raw luminescence values in treatment groups to the median luminescence value in the vehicle-treated group. Statistical analysis was conducted using an unpaired two-sided Student t test using Welch's correction to determine significance.

The statistical significance of PPP2R2C expression in gene expression microarray data and tissue microarray (TMA) nuclear intensity staining was conducted using a Student t test. Multivariate analysis assessing the association between PPP2R2C staining intensity and patient survival data was conducted using the Proc-Mixed procedure in SAS (SAS Institute) adjusting for the random effects of preoperative prostate-specific antigen (PSA) and tumor stage. The mean nuclear staining intensity (MNI) value was calculated by averaging the values for all TMAs cores derived from each individual patient. Kaplan–Meier survival estimates were generated with the Proc-Lifetest procedure in SAS using a log-rank significance threshold of P < 0.05. Patients were grouped on the basis of high PPP2R2C expression in primary tumors (MNI ≥ 1) and low PPP2R2C expression in primary tumors (MNI < 1).

**Quantitative real-time PCR**

RNA was isolated from cells 24 hours posttransfection using the RNAeasy Kit (Qiagen Inc.) and cDNA was generated with Superscript II (Life Sciences) as per protocol. qRT-PCR reactions were carried out in triplicate using an Applied Biosystems 7900 sequence detector with SYBR Green PCR master mix (Life Technologies). Primers were designed using PrimerQuest (IDT), and all reactions were normalized to the housekeeping gene (complete Mini; Roche Diagnostics). Protein was quantified per protocol using a bicinchoninic acid assay (Thermo Scientific). Normalized cell lysates were loaded onto a 12% NuPAGE Bis–Tris gel (Life Sciences) in MES [2-(N-Morpholino)ethanesulfonic acid] buffer. Protein was transferred to nitrocellulose membranes using a semi-dry transfer apparatus and Tris/CAPS buffer. Immunoblots were probed with primary antibodies targeting AKT (Cell Signaling Technology; #2020), phospho-AKT (Cell Signaling Technology; #4060), Erk1/2 (Sigma; M5670), diphosphorylated-Erk1/2 (Sigma; M9692), PPP2R2C (Abnova; clone OF3) and antiactin antibody (Santa Cruz Biotechnology; sc-1616) as a loading control.

**Protein collection and immunoblotting**

Protein was collected from tissue culture by lysing adherent cells with a cell lysis buffer (1.5 mol/L urea, 1% SDS, 1% NP-40, 2% Tween-20, 250 nmol/L NaCl, and PBS) supplemented with 1× phosphatase inhibitors (PhosStop; Roche Diagnostics) and a 1× protease inhibitor cocktail (Complete Mini; Roche Diagnostics). Protein was quantified per protocol using a bicinchoninic acid assay (Thermo Scientific). Normalized cell lysates were loaded onto a 12% NuPAGE Bis–Tris gel (Life Sciences) in MES [2-(N-Morpholino)ethanesulfonic acid] buffer. Protein was transferred to nitrocellulose membranes using a semi-dry transfer apparatus and Tris/CAPS buffer. Immunoblots were probed with primary antibodies targeting AKT (Cell Signaling Technology; #9272), phospho-AKT (Cell Signaling Technology; #4058), Erk1/2 (Sigma; M5670), diphosphorylated-Erk1/2 (Sigma; M9692), PPP2R2C (Abnova; clone 6D1), Src (Cell Signaling Technology; #2110), and phospho-Src (Cell Signaling Technology; #2101). Horseradish peroxidase–conjugated secondary antibodies (Thermo Scientific) were used in conjugation with Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) to visualize protein targets. Membranes were then stripped for 15 minutes in Stripping Buffer (Thermo Scientific) and reprobed with antiactin antibody (Santa Cruz Biotechnology; sc-1616) as a loading control.

**Tissue microarray construction**

Prostate specimens were collected from a cohort of 100 men who underwent radical prostatectomy at Virginia Mason Medical Center (Seattle, WA) between 1954 and 1997 (Supplementary Table S1). Tissue specimens were formalin-fixed and embedded in paraffin following surgical resection. Patients were monitored for both biochemical recurrence (PSA > 0.2 ng/mL following radical prostatectomy) and pathologic recurrence (pelvic disease, regional lymph node metastasis, distant metastasis, or prostate cancer-specific mortality (PCS)). Tissue diagnosis or overt radiographic signs confirmed the presence of disease recurrence. To classify patients as developing PCSM, death certificates were obtained and prostate cancer had to be the first listed cause of death. Thirty-four patients were identified with known adverse outcomes of either distant metastasis or
Immunohistochemistry

FFPE TMAs were deparaffinized and rehydrated. Three percent hydrogen peroxide was used to quench endogenous tissue peroxidases and an avidin/biotin blocking system (Dako) was used to quench endogenous avidin/biotin. Antigen retrieval was conducted with 10 mmol/L citrate buffer, pH 6.0 in a pressure cooker. Tissue was blocked with 1% hydrogen peroxide was used to quench endogenous tissue peroxidases and an avidin/biotin blocking system (Dako) was used to quench endogenous avidin/biotin. Tissue blocks were counterstained with Meyer’s hematoxylin (Dako). Immunohistochemistry was conducted with 3,3′-Diaminobenzidine (DAB; Dako) was used as the chromogen and the TMA was counterstained with Meyer’s hematoxylin (Dako). PPP2R2C nuclear staining was assessed by a genitourinary pathologist and graded on a 0–3 scale, with a score of 3 representing intense nuclear staining.

Results

siRNA screen identifies modifiers of castration-resistant prostate cancer proliferation

To identify genes capable of promoting the growth of prostate cancer cells in the absence of exogenous AR ligands, we conducted a HTRS using 2 androgen-sensitive prostate cancer cell lines; LNCaP and VCaP. Briefly, cells were cultured in CSS to simulate ADT. Optimal transfection conditions were determined as previously described (21, 22). A library of siRNAs designed to inhibit the translation of 6,650 individual genes was used in an arrayed screening strategy whereby a pool of 3 siRNAs targeting each specific gene was separately introduced into replicate cell cultures (1 gene per well) in 384-well culture plates. After 96 hours of growth, cell numbers were estimated using the Cell Titer-Glo luminescence reagent. Raw luminescence signal intensity from the HTRS screen was transformed into Z scores within each plate, and median standardized Z scores for each gene were used in downstream analysis. To prioritize genes for further study, we arbitrarily set a significance threshold of $Z > 1.96$. At this cutoff point, suppression of 380 unique genes in LNCaP and 240 unique genes in VCaP induced cell growth in the absence of exogenous androgens (Fig. 1), of which 40 induced growth in both cell lines (Table 1). Of the 40 genes whose suppression induced growth in both LNCaP and VCaP cells, 2 encoded components of the PP2A serine/threonine phosphatase complex: PPP2R2C and PPP2R1A.

PP2A subunit expression is reduced in primary and metastatic prostate cancers

To prioritize HTRS hits exhibiting potential tumor-suppressor expression patterns, we cross-referenced the screen results with transcript abundance levels determined by microarray measurements of laser-capture microdissected benign prostate epithelia (n = 15), ADT-resistant primary prostate tumors (n = 14), and CRPC metastases (n = 54). Out of the 40 proliferation-suppressor genes identified in the in vitro HTRS, 14 genes were significantly downregulated in primary and metastatic CRPC samples compared with benign prostate epithelia, including PPP2R2C and PPP2R1A (Fig. 2). Specifically, the mean expression of PPP2R2C in these primary and metastatic cancers was 3.85-fold lower ($P = 0.034$) and 5.69-fold lower ($P < 0.001$) than benign prostate epithelia, respectively. PPP2R1A expression was also decreased in metastatic prostate tumors (1.55-fold; $P < 0.001$).
We next evaluated HTRS experimental results and human prostate gene expression data for evidence of other PP2A components behaving as tumor suppressors. Previous studies have reported that suppression of the PP2A constituents PPP2CA and PPP2R2A can promote CRPC cell growth (15, 16). However, transcripts encoding these PP2A subunits were not downregulated in the primary prostate cancers we evaluated (Fig. 3A). Furthermore, transcripts encoding PPP2CA were significantly higher in metastatic CRPC (1.9-fold; *P* < 0.001; Fig. 3A). In the HTRS experiments, siRNAs targeting PPP2CA or PPP2R2A did not induce significant castration-resistant growth in either LuCAP or VCaP cells (Fig. 3B).

As 4 of 16 PP2A subunits tested in the HTRS experiments induced AR ligand-independent proliferation in at least one cell line (Fig. 3B), we sought to further assess the mechanism(s) by which PP2A activity contributes to this phenotype. We selected PPP2R2C for further investigation based on the findings that PPP2R2C induced significant androgen depletion-resistant proliferation in both cell lines evaluated, and PPP2R2C was the HTRS hit found to be most downregulated in castration-resistant

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metastatic prostate cancer compared with benign epithelium (Fig. 2).

**Knockdown of PPP2R2C promotes prostate cancer growth in the absence of androgens without inducing AR activity in vitro**

Because the siRNA screens were conducted using pools of 3 siRNAs targeting each gene, we evaluated individual siRNA efficacy by transfecting LNCaP and VCaP with the deconvoluted pool of PPP2R2C siRNAs, designated siRNA #1-3. Suppression of PPP2R2C by gene-specific siRNA was confirmed by qRT-PCR and correlated with assays of cell proliferation. PPP2R2C knockdown by si#2 and si#3 were confirmed at the protein level in both cell lines (Fig. 4A). Scrambled control siRNAs (siUNI) did not alter PPP2R2C expression or influence cell proliferation (Fig. 4B). The positive control for cell death, siKiff1, substantially reduced the number of tumor cells in both LNCaP and VCaP experiments (Fig. 4B). As expected, exposing LNCaP or VCaP cells to androgen (R1881) stimulated proliferation and induced the AR target genes PSA, FKBP5, TMPRSS2, and ERG (in VCaP; Fig. 4B–D). siRNA#1 suppressed PPP2R2C transcripts by 10-fold in both cell lines, but did not influence cell proliferation in either line. However, both siRNA #2 and siRNA #3 reduced PPP2R2C mRNAs in LNCaP by 11.1- and 4.2-fold ($P < 0.001$), respectively (Fig. 4B and C), and induced cell proliferation in androgen-depleted medium by 42% (siRNA #2; $P < 0.001$) and 72% (siRNA #3; $P < 0.001$). This increase in cell number approximated the influence of adding the androgen (Fig. 4B). In VCaP cells, PPP2R2C transcripts were reduced 6.7-fold by siRNA #2 and 4.2-fold by siRNA #3 ($P < 0.001$; Fig. 4D), and these siRNAs increased cell proliferation by approximately 33% in androgen-depleted growth conditions ($P < 0.001$; Fig. 4B). Knockdown of PPP2R2C in the presence of androgens did not significantly increase growth in either cell line (Supplementary Fig. S1). PPP2R2C suppression did not alter AR or PSA expression in either cell line (Fig. 4C and D). Suppression of PPP2R2C with siRNA #2 in LNCaP cells did slightly decrease the expression of FKBP5 (1.4-fold; $P = 0.02$) and TMPRSS2 (1.3-fold; $P = 0.02$) and resulted in a 1.6-fold increase in ERG expression ($P = 0.01$; Fig. 4C). Suppression of PPP2R2C with siRNA #3 in VCaP resulted in decreased TMPRSS2 expression (1.2-fold; $P = 0.01$; Fig. 4D). PPP2R2C downregulated with si#3 in LNCaP and si#2 in VCaP did not affect androgen-regulated gene expression. These data indicate that the growth advantages attained from PPP2R2C knockdown were not mediated by an increase in canonical AR transcriptional activity.
To further assess the potential role of the AR in PPP2R2C-mediated prostate cancer cell growth in the context of androgen depletion, LNCaP and VCaP cells were treated with the deconvoluted pool of PPP2R2C siRNAs (si#1-3), in addition to a scrambled control (siUNI) and a positive control for transfection (siKif11). siUNI + 1 nmol/L R1881 is a positive control for androgen-induced cell growth and gene expression. A. Western immunoblot analysis of PPP2R2C protein with siRNAs targeting PPP2R2C. B. growth of LNCaP and VCaP cells in androgen-depleted medium is induced by PPP2R2C-targeted siRNA #2 and siRNA #3, but not siRNA #1. C and D. qRT-PCR analysis of cDNA collected from LNCaP (C) and VCaP (D) shows successful suppression of PPP2R2C expression. PPP2R2C knockdown does not increase the expression of AR or AR-regulated genes (PSA, FKBP5, TMPRSS2, and ERG). Cotreatment with nontargeting siRNA + 1 nmol/L R1881 induced AR-regulated gene expression. Statistical comparisons of gene expression were conducted between siUNI and gene-specific siRNA within each cell line. E and F. LNCaP (E) and VCaP (F) were transfected with nontargeting siRNA or siRNA targeting PPP2R2C and cotreated with 5 μmol/L MDV3100. Raw luminescence values in each cell line were normalized to siUNI. Statistical comparisons between siRNA-targeting PPP2R2C and siUNI were conducted within each cell line.

PPP2R2C knockdown does not mediate proliferation through activation of SRC, PI3K, or ERK1/2 signal transduction in prostate carcinoma

Previous experimentation in nonepithelial cancer models indicate that PPP2R2C may negatively regulate the activity of the v-src sarcoma viral oncogene homolog SRC (23). As SRC has been postulated to promote CRPC growth (24, 25), we hypothesized that PPP2R2C loss may promote...
castration-resistant growth in LNCaP and VCaP cells by modulating SRC phosphorylation and consequent activity. However, knockdown of PPP2RC did not result in SRC phosphorylation (Fig. 5A and B) and treatment with the SRC inhibitor dasatinib failed to inhibit castration-resistant growth induced by PPP2R2C knockdown (Fig. 5C and D). Therefore, these experiments indicate that androgen depletion-resistant growth of LNCaP and VCaP cell lines following PPP2R2C knockdown is not mediated through enhanced SRC activity.

We next evaluated the influence of PPP2R2C in other signal transduction pathways postulated to influence CRPC growth. Specifically, we investigated the phosphorylation of ERK1/2 and protein kinase B/AKT (AKT; refs. 8, 26). Depletion of PPP2R2C did not result in observable changes in the phosphorylation of ERK1/2 when compared with the scrambled control siRNA. AKT phosphorylation was slightly decreased in VCaP, but was unchanged in LNCaP (Fig. 5A and B). To confirm that PPP2R2C knockdown did not induce growth through PI3K or ERK1/2 activation, cells were cotreated with pharmacologic inhibitors of these pathways and siRNAs targeting PPP2R2C. Inhibition of PI3K with LY294002, or mitogen-activated protein/extracellular signal–regulated kinase (MEK) with U0126, failed to abrogate growth induction mediated by PPP2R2C knockdown (Fig. 5E–H). Therefore, it seems that PPP2R2C knockdown
influences signaling pathways capable of promoting prostate cancer cell growth that are distinct from PI3K, ERK1/2, or SRC in the setting of androgen depletion.

**Decreased PPP2R2C expression is primary prostate cancers is associated with disease relapse and prostate cancer-specific mortality**

Because PPP2R2C downregulation promotes castration-resistant growth, we hypothesized that PPP2R2C expression might influence long-term disease outcome in men with prostate carcinoma. To explore this hypothesis, we conducted immunohistochemical (IHC) staining for PPP2R2C on radical prostatectomy tissues from a cohort of 100 patients with documented long-term clinical outcomes that included biochemical relapse, local recurrence, distant metastases, and PCSM (Supplementary Table S1). A range of PPP2R2C protein expression was noted between individual cores, yet staining within each tumor core was largely homogenous. PPP2R2C staining of LNCaP and VCaP cell lines also showed nuclear expression (Supplementary Fig. S2). Each TMA core was categorized on the basis of IHC nuclear intensity according to a scale from 0–3, with a score of 0 representing no PPP2R2C staining and a score of 3 representing high-intensity nuclear staining (Fig. 6A–D). We analyzed the impact of nuclear PPP2R2C expression on prostate cancer morbidity and mortality using the Proc Mixed model in SAS (SAS 9.3) adjusted for patient pre-surgical serum PSA levels and tumor stage at the time of prostatectomy. Lower PPP2R2C staining was significantly correlated with the risk of biochemical relapse ($P < 0.01$) and the development of distant metastases ($P < 0.001$). We also observed that PCSM was correlated with low PPP2R2C protein expression ($P = 0.048$; Table 2). Kaplan–Meier survival curves for patients with tumors expressing high PPP2R2C (MNI $\geq 1$) and those with low PPP2R2C (MNI < 1) revealed a survival advantage in those patients whose tumors expressed high levels of PPP2R2C (log-rank test $P = 0.045$; Fig. 6E).

**Discussion**

The development and clinical use of increasingly potent therapeutics targeting the AR and androgenic steroid production in prostate cancer (4, 5) is likely to select against those tumor cells relying on AR-mediated pathways for survival. Therefore, identifying AR pathway-independent prostate cancer growth and survival mechanisms will become increasingly relevant in clinical disease. Here, we provide evidence that decreased expression of the regulatory PP2A subunit, PPP2R2C, promotes CRPC growth. Importantly, growth induction was not mediated by the upregulation of the AR or androgen-regulated genes, and growth was not inhibited by treatment with the potent AR antagonist MDV3100. These data strongly suggest that PPP2R2C loss activates growth and survival pathways that bypass traditional approaches to suppress prostate cancer via the AR. Therefore, we expected to observe PPP2R2C loss in a subset of clinical prostate tumors. Multivariate regression analysis showed a statistically significant association between decreased PPP2R2C expression, disease relapse, and increased PCSM. Kaplan–Meier survival analysis indicated that low PPP2R2C expression in primary tumors is associated with decreased long-term survival. To our knowledge,

**Table 2. Multivariate analysis of PPP2R2C staining and patient outcomes**

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>PPP2R2C MNI</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical relapse</td>
<td>No 2.25</td>
<td>$=0.006$</td>
</tr>
<tr>
<td></td>
<td>Yes 1.74</td>
<td></td>
</tr>
<tr>
<td>Distant metastases</td>
<td>No 2.32</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Yes 1.55</td>
<td></td>
</tr>
<tr>
<td>PCSM</td>
<td>No 2.21</td>
<td>$=0.048$</td>
</tr>
<tr>
<td></td>
<td>Yes 1.79</td>
<td></td>
</tr>
</tbody>
</table>
this is the first evidence suggesting that PPP2R2C loss may influence cancer-specific mortality.

PPP2A is a known tumor suppressor and loss of PPP2A phosphatase activity or altered substrate specificity has been shown to occur frequently in several human malignancies (10). Decreased PPP2A function is sufficient to promote castration-resistant growth of the LNCaP cell line, and studies of the castration-resistant LNCaP derivative C4-2b, have reported lowered levels of the catalytic subunit PPP2CA (17). However, complete loss of PPP2CA expression can be detrimental to cancer cell growth and result in cell death (13), suggesting that PPP2CA expression plays a dual role in promoting survival and negatively regulating proliferation. Considering that the SV40ST oncogene promotes cell transformation by inhibiting B-subunit binding without initiating apoptosis (14), it is likely that loss of select PPP2A B-subunits could alter PPP2A substrate specificity and promote tumorigenesis while avoiding the apoptotic effect of total PPP2CA loss. Knockdown of individual PPP2A regulatory B-subunits has shown differential effects on cell transformation (10, 13), an effect replicated in the HTRS experiments conducted in this study. Investigation into transforming B-subunit loss has shown patterns of growth pathway deregulation similar to those observed with inhibition of PPP2A catalytic activity and those induced by SV40ST oncogene expression (13).

The effect of PPP2R2C expression on PPP2A substrate specificity is largely unknown. However, PPP2R2C has been shown to negatively regulate the activity of the SRC oncogene in osteosarcoma cells (23). Surprisingly, PPP2R2C downregulation did not alter SRC pathway activity in prostate cancer cells. This discrepancy may be due to tissue-specific differences in subcellular localization of the PPP2A complex. In osteosarcoma cells, investigators detected perinuclear expression of PPP2R2C (23). In contrast, PPP2R2C showed distinct nuclear localization in prostatic tissue. Tissue-specific variations in the subcellular localization of other PPP2A B-regulatory subunits are widely reported; for example, PPP2R2A is detected in the cytoplasm of neural extracts (27), but expression is nuclear in the Jurkat cell line and rat epidermis (28, 29).

Further mechanistic studies are necessary to identify specific PPP2A targets mediated by PPP2R2C expression in prostate carcinoma. Because PP2A directly regulates the activity of more than 50 kinases and other known proto-oncogenes (10, 11), this will likely require extensive proteomics-based experiments with an emphasis on deregulated survival and growth pathways found in clinical CRPC. The promotion of CRPC growth via decreased PPP2R2C expression provides an important example, supported by in vivo patient outcomes, of prostate cancer survival pathways that do not function through increased AR activity, but instead through the deregulation of existing signal transduction pathways. The identification of pharmacologic inhibitors of PPP2R2C complexes, or the growth pathway(s) activated by PPP2R2C loss may prove useful as a therapeutic in men with CRPC, or as neoadjuvant therapy in men with downregulated PPP2R2C in primary disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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