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Cancer Research

Human RecQL4 Helicase Plays Critical Roles in Prostate Carcinogenesis

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Abstract

Prostate cancer is the second leading cause of cancer-associated deaths among men in the western countries. Here, we report that human RecQL4 helicase, which is implicated in the pathogenesis of a subset of cancer-prone Rothmund-Thomson syndrome, is highly elevated in metastatic prostate cancer cell lines. Increased *RecQL4* expression was also detected in human prostate tumor tissues as a function of tumor grade with the highest expression level in metastatic tumor samples, suggesting that *RecQL4* may be a potential prognostic factor for advanced stage of prostate cancer. Transient and stable suppression of *RecQL4* by small interfering RNA and short hairpin RNA vectors drastically reduced the growth and survival of metastatic prostate cancer cells, indicating that *RecQL4* is a prosurvival factor for prostate cancer cells. *RecQL4* suppression led to increased poly(ADP-ribose) polymerase (PARP) synthesis and *RecQL4*-suppressed prostate cancer cells underwent an extensive apoptotic death in a PARP-1–dependent manner. Most notably, *RecQL4* knockdown in metastatic prostate cancer cells drastically reduced their cell invasiveness *in vitro* and tumorigenicity *in vivo*, showing that *RecQL4* is essential for prostate cancer promotion. Observation of a direct interaction of retinoblastoma (Rb) and E2F1 proteins with *RecQL4* promoter suggests that Rb-E2F1 pathway may regulate *RecQL4* expression. Collectively, our study shows that *RecQL4* is an essential factor for prostate carcinogenesis.
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Introduction

The prototypical *Escherichia coli* RecQ protein acts as a suppressor of illegitimate recombination and *RecQ* mutants exhibit genomic instability due to improper resolution of DNA secondary and tertiary structures arising during replication and recombination (1). Human cells express five distinct *RecQ* homologues, three of which are associated with autosomal recessive diseases characterized by cancer susceptibility (2, 3): Bloom syndrome, Werner syndrome, and Rothmund-Thomson syndrome (RTS). Mutations in *RecQL4* are linked to three autosomal recessive disorders (RTS, RAPADILINO, and Baller-Gerold syndrome; ref. 4), and the RecQL4 protein shares 40.8% homology with the *E. coli* RecQ protein (see ref. 5 and references therein).

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Human RecQ helicases play diverse roles in DNA metabolism (3, 6). RecQL1 promotes chromosome stability and integrity (7), WRN and BLM participate in organizing the DNA replication complex (8), and WRN helicase plays a role in RNA polymerase II–mediated basal transcription in human cells (9). Although the precise biological role of *RecQL4* awaits further investigations, some studies implicate RecQL4 in DNA replication and DNA repair (10, 11). Interaction of RecQL4 with proteins involved in genome surveillance was recently reported (12). Xu and Liu (13) provided initial evidence for the DNA unwinding activity of RecQL4 *in vitro* in the presence of ssDNA, and the activity has been subsequently shown for both ssDNA and dsDNA (14). It is currently unclear whether or not cancer cells exhibit deregulation of RecQ helicases, and if so whether deregulated RecQ helicase activities initiate and promote cancer development processes.

Prostate cancer incidence is increasing at an alarming rate, now representing 33% of new cases of male cancers. Gene deletion and amplification events involving chromosome 8q are intimately associated with prostate carcinogenesis (15–19), although the critical genes in this region have not been extensively characterized. Chromosome 8q amplification was reported in metastatic and recurrent prostate cancers (20–22). Chromosome 8q (11–24) gain is not only observed in prostate cancers but also in cervical cancer (23, 24), breast cancer (25), and colorectal cancer (26), indicating its intimate association with tumorigenesis. *RecQL4* imbalance in expression has recently been reported in 18 sporadic osteoblastoma samples (27). Here, we report that

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human RecQL4 helicase localized at 8q24.3 is highly expressed in metastatic human prostate cancer cells and tumor tissues. *RecQL4*-suppressed PC3 cells displayed drastically reduced cell invasion potential *in vitro* and tumorigenic potential *in vivo*. These findings suggest that *RecQL4* may play a critical role in prostate carcinogenesis. Furthermore, the high level of *RecQL4* expression in metastatic prostate cancer samples suggests that *RecQL4* may be a novel biomarker for advanced stage of prostate cancer.

Materials and Methods

Cell lines and prostate tumor tissues

Human primary prostate epithelial cells (PrEC) were procured from Lonza, Inc. Immortalized (RWPE1) and metastatic prostate cancer cells lines (CWR22, DU145, LNCaP, PC3, and RWPE2) were procured from American Type Culture Collection. All the cell lines were authenticated based on viability, recovery, growth, and morphology by the suppliers. Human prostate tissue panel array slides (PR951 and PR751) were procured from US Biomax, Inc.

Fluorescence *in situ* hybridization and immunohistochemistry

Procedures for metaphase chromosome preparation, fluorescence *in situ* hybridization (FISH), and multicolor FISH were essentially the same as described before (28). FISH was performed using multicolor-band probe specific for chromosome 8 and a spectrum orange-labeled bacterial artificial chromosome (BAC) probe (RP11-374B7, Open Biosystems) corresponding to the proximal region of chromosome 8q24.3 locus. Human M-band and M-FISH probes were obtained from MetaSystems.

Rabbit polyclonal antibodies specific for NH_2 terminal and COOH terminal regions of RecQL4 were generously provided by Dr. Igor Stagljar (University of Toronto). RecQL4 expression in the prostate tumor tissue arrays was analyzed by immunohistochemistry using a COOH terminal antibody. Detailed procedures for immunostaining, image acquisition, and quantification are given in Supplementary Data.

Suppression of *RecQL4* in prostate cancer cells

Immortalized normal prostate epithelial cells (RWPE1) and metastatic prostate cancer cell lines (DU145, LNCaP, and PC3) in exponential growth phase were transfected with 25 to 100 nmol/L of either control scrambled small interfering RNA (siRNA; Santa Cruz Biotechnology) or *RecQL4*-specific siRNA (Santa Cruz Biotechnology). Cells were transfected using Lipofectamine RNAi Max (Invitrogen). RecQL4 expression in the total cellular proteins isolated 72 hours after transfection was monitored by Western blot using either COOH terminal or NH_2 terminal antibody. Empty (TR20002), scrambled (TR30003), and four different *RecQL4* specific short hairpin RNA (shRNA) vectors (TI339521, TI339522, TI339523, and TI339524) were procured from Origene. Transfection of PC3 cells with different shRNA vectors (2–4 μg) were performed using Lipofectamine 2000 (Invitrogen) essentially following the manufacturer's protocol.

Cell proliferation, survival, cell invasion, and tumorigenicity of RecQL4-suppressed prostate cancer cells

Proliferation of prostate cancer cells transfected with control and *RecQL4*-specific siRNA was analyzed by fluorescence-based CyQuant assay (Invitrogen) essentially as described before (29). To measure cellular invasion, InnoCyte cell invasion kit (EMD Biosciences, Inc.) was used following the manufacturer's instructions. To determine whether *RecQL4* suppression also leads to reduced tumorigenicity *in vivo*, 6×10^6 cells from each of the four cell lines [nontransfected PC3, empty vector–transfected clone C5, and two *RecQL4* shRNA vector–transfected clones (C6 and C4)] were injected into nude mice. At least five mice were used for each treatment. The entire experimental animal protocol and procedure were in compliance with the guidelines of Institutional Animal Care and Use Committee of Columbia University Medical Center. Tumor growth and size (mm^3) were monitored for up to 4 weeks.

Effect of histone deacetylase inhibitor Trichostatin A on *RecQL4* expression

Immortalized prostate epithelial cells (RWPE1) and the three metastatic prostate cancer cell lines (DU145, LNCaP, and PC3) in exponential growth phase were treated with 0.5 and 1 μmol/L of Trichostatin A (TSA; Sigma-Aldrich) for 48 hours. RecQL4 expression was monitored at the mRNA, and protein levels by reverse transcription-PCR (RT-PCR), quantitative real-time PCR, and Western blot analyses. Forward and reverse primer sequences for *RecQL4* are given in Supplementary Data. Antibodies were procured from Santa Cruz Biotechnology [cyclin A, cyclin E, Cdk2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and α-tubulin], Millipore (γ-H2AX), Cell Signaling Technology [phospho-retinoblastoma (Rb) $Ser^{807/811}$], and Invitrogen (phospho-Rb T^{821}).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed in HeLa cells using a standard protocol essentially as described before (30). Antibodies for Rb and E2F1 were procured from Santa Cruz Biotechnology and Novus Biologicals, respectively.

Results

Human RecQL4 helicase expression is elevated in human prostate cancer cell lines

RecQL4 expression was assessed by RT-PCR and quantitative real-time PCR in primary (PrEC) and immortalized (RWPE1) human prostate epithelial cell lines, as well as in four human prostate cancer cell lines (CWR22, DU145, LNCaP, and PC3). *RecQL4* expression was markedly increased in all the metastatic prostate cancer cell lines compared with primary (PrEC) and immortalized human prostate epithelial cells

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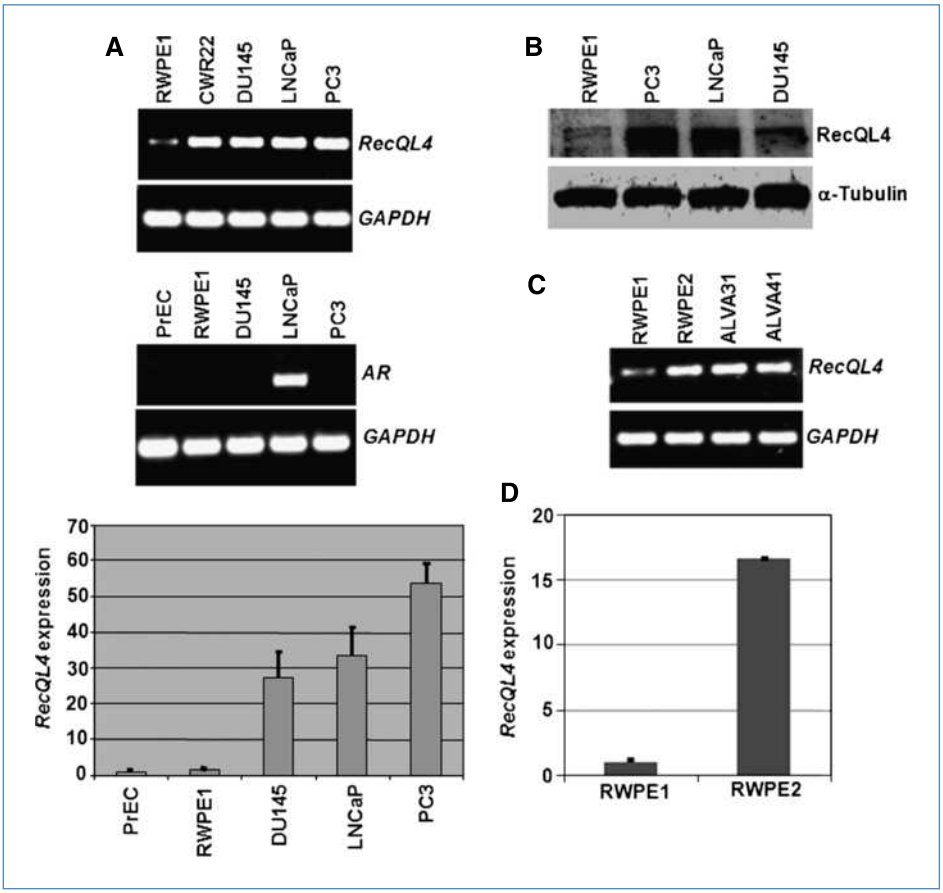

Figure 1. *RecQL4* expression analyses at the mRNA and protein levels in normal and prostate cancer cell lines of human origin. A, RT-PCR analysis of *RecQL4* and androgen receptor (*AR*) expression in normal immortalized human prostate epithelial cells (RWPE1) and four metastatic prostate cancer cell lines. *GAPDH* was used as a loading control. Quantitative real-time PCR analysis of *RecQL4* expression in primary (PrEC), SV40 immortalized (RWPE1), and metastatic prostate cancer cell lines (DU145, LNCaP, and PC3). *RecQL4* expression in normal and cancer cell lines was normalized to β-actin. Bars, SEM. B, Western blot analysis of RecQL4 expression in normal and prostate cancer cell lines. α-Tubulin was used as a loading control. C, RT-PCR analysis of RecQL4 expression in nontumorigenic (RWPE1), tumorigenic (RWPE2), and prostate cancer cell lines (ALVA31 and ALVA41). D, quantitative real-time PCR analysis of *RecQL4* in nontumorigenic (RWPE1) and tumorigenic (RWPE2) cells. Error bars, SEM.

(RWPE1; Fig. 1A) in an androgen-independent manner (Fig. 1A). *RecQL4* transcript detected by real-time PCR (Fig. 1A) correlated well with RecQL4 protein in the prostate cancer cells (Fig. 1B). To examine whether or not elevated *RecQL4* expression is linked with malignant transformation process, *RecQL4* expression was analyzed in the moderately tumorigenic RWPE2 cells that were derived from the parental nontumorigenic RWPE1 cells by transformation with Ki-ras using the Kirsten murine sarcoma virus (31). Strikingly, *RecQL4* expression was ~15-fold higher in tumorigenic RWPE2 cells than RWPE1 cells (Fig. 1C and D). *RecQL4* expression was found elevated in all the six human prostate cancer cell lines: CWR22, DU145, LNCaP, PC3, ALVA31, and ALVA41 (Fig. 1A and C). Consistent with elevated *RecQL4* expression, spectrum orange-labeled BAC probe proximal to *RecQL4* locus (8q24.3) revealed three, four, and six hybridization spots in DU145, LNCaP, and PC3 cell lines, respectively (Supplementary Fig. S1).

RecQL4 expression is enhanced in human prostate tumor tissues

RecQL4 expression was next investigated in human prostate panel arrays PR951 and PR751. PR951 array included 8 normal prostate, 36 prostate adenocarcinoma with different Gleason scores, and 4 metastatic adenocarcinoma. PR751 array included 7 benign prostatic hyperplasia (BPH), 4 prostatic intraepithelial neoplasias (PIN), and 64 prostate adenocarcinoma with Gleason scores from 2 to 10. In general, intense RecQL4 protein staining was mainly detected in the nucleoplasmic regions. The integrated absorbance (IA) was used to categorize the staining intensity for RecQL4 as low, medium, and high. Representative images of normal and prostate tumor tissue sections are shown in Fig. 2A and B, and quantitative results are summarized in Table 1 and Fig. 2C. The highest level of RecQL4 expression was detected in all the metastatic prostate adenocarcinoma samples (Fig. 2A, IV–VI), and RecQL4 immunoreactivity somewhat correlated with increasing Gleason scores in the prostate tumor samples. Thirty prostate tumor tissue samples of 48 in the PR951 array had PSA values ranging from 0.5 to 161 ng/mL, but the PSA value was unavailable for the samples in the PR751 array. In general, PSA values of the samples did not seem to correlate with Gleason scores (2–10). However, PSA value and RecQL4 staining intensity correlated for 12 of 30 samples. Two of four PIN samples and two of seven BPH samples showed a medium level of RecQL4 staining intensity in prostatic ducts, ductules, and acini (Fig. 2B). The cumulative average IA values obtained for normal, malignant, and metastatic tumor tissue samples are presented in Fig. 2C. Importantly, the aggregate data show that higher RecQL4 expression correlates with higher prostate cancer grade/severity in this set of 123 tissue samples.

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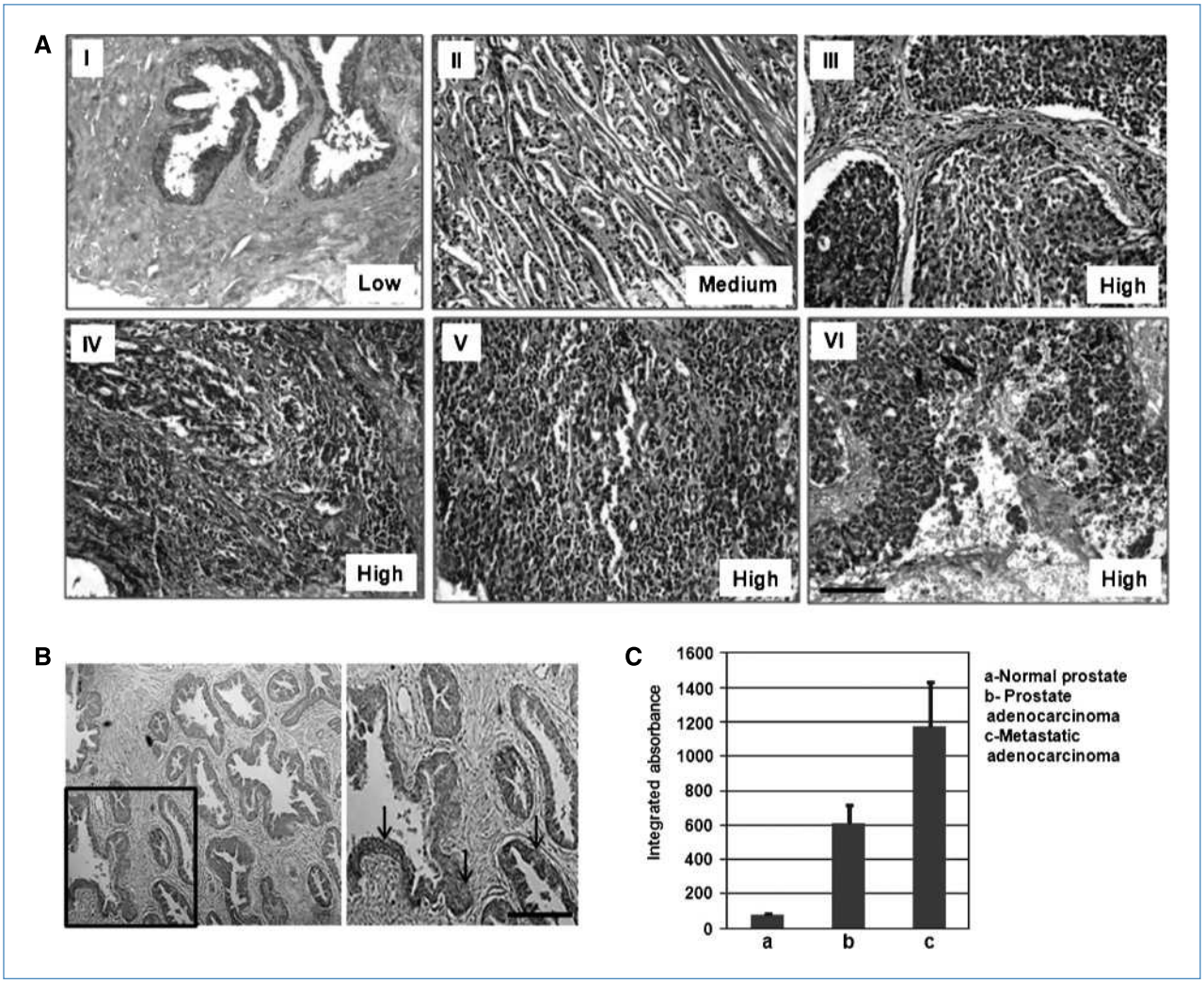

Figure 2. A, immunohistochemical analysis of RecQL4 expression in normal (I), malignant (II and III), and metastatic prostate tumor tissues (IV–VI). Prostate tissue panel arrays (PR951 and PR751) purchased from US Biomax, Inc., were used. Malignant tumor samples shown in II (age, 67 y; Gleason score, 7; PSA, 37.3 ng/mL) and III (age, 63 y; Gleason score, 10; PSA, not determined) illustrate that the RecQL4 expression increases with increasing tumor grade. Samples: IV, 61-y-old male; metastatic adenocarcinoma to bone; V and VI, 65-y-old, metastatic adenocarcinoma to bone and abdominal wall, respectively. B, staining pattern of a PIN sample (age, 50 y; Gleason grade III) is shown in the left bottom. Arrows indicate the regions of positive RecQL4 staining. C, the cumulative average value of IA obtained for all the normal, malignant, and metastatic samples are given. Bars, SEM. Scale bar, 50 μm.

RecQL4 gene silencing inhibits proliferation potential of prostate cancer cells

To verify the importance of *RecQL4* in prostate cancer cell proliferation and survival, siRNA-mediated *RecQL4* gene silencing studies were performed in normal immortalized (RWPE1) and metastatic prostate cancer cell lines. Transfection of *RecQL4*-specific siRNA reduced the expression of RecQL4 by 70% to 90% in all the cell lines. Representative pictures of RecQL4 knockdown in prostate cancer cell lines after transfection with 50 nmol/L of control and *RecQL4*-specific siRNA are shown in Fig. 3A and B. Proliferation measured using the CyQuant assay kit (Invitrogen; ref. 29) showed a modest inhibition in proliferation in RWPE1 cells transfected with 10 nm (9.8%) 25 nmol/L (20.2%) of *RecQL4*-specific siRNA. In contrast, PC3 cells transfected with 10, 25, and 50 nmol/L of *RecQL4*-specific siRNA showed 42.1%, 48.3%, and 51.4% inhibition in proliferation, respectively (Fig. 3C). These findings suggest that *RecQL4* depletion affected the proliferation more profoundly in metastatic prostate cancer cells than in normal prostate epithelial cells.

RecQL4 gene silencing reduces prostate cancer cell viability

The reduced proliferation potential in *RecQL4*-silenced PC3 cells prompted us to examine the effects of *RecQL4* ablation on cell cycle progression and cell death in the three prostate cancer cell lines. For this purpose, cells in exponential growth phase (RWPE1, DU145, LNCaP, and PC3) were transfected with 25 to 100 nmol/L of either control siRNA or *RecQL4*-specific

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siRNA and subjected to cell cycle analysis 72 hours after transfection. Whereas cell cycle progression was hardly affected by *RecQL4* knockdown in RWPE1 cells, all the prostate cancer cell lines transfected with *RecQL4*-specific siRNA showed a dose-dependent increase in the sub-G_1 fraction (Fig. 3D). The proportion of G_1 cells also decreased as a function of increasing *RecQL4* siRNA concentration in all the prostate cancer cell lines but most notably in LNCaP and PC3 cell lines (Fig. 3D). The fraction of G_2-M phase cells was also reduced in PC3 cells at the highest dose of *RecQL4* siRNA (Fig. 3D). Induction of apoptotic markers [poly(ADP-ribose) polymerase I (PARPI), Bax, and apoptosis inducing factor (AIF)] was next analyzed in *RecQL4*-suppressed PC3 cells. Consistent with the cell cycle data, cleaved PARP-1, Bax, and AIF proteins showed a dose-dependent enrichment in *RecQL4* siRNA-transfected PC3 cells but not in RWPE1 cells (Fig. 4A). Collectively, these results point out that *RecQL4* is critical not only for maintaining cell cycle progression but also for survival in prostate cancer cells.

Stable *RecQL4* knockdown increases spontaneous DNA strand break accumulation in metastatic prostate cancer cells

Effects of *RecQL4* suppression on clonogenic survival was subsequently analyzed in PC3 cells through the use of *RecQL4* targeting shRNA vectors (TI339521 and TI339524) together with empty (TR20003) and scrambled shRNA (TR30003) control vectors (Origene Technology). Transient *RecQL4* suppression by both shRNA vectors reduced the clonogenic survival of PC3 cells (Fig. 4B). To extend these studies, several stably transfected (empty, scrambled, and RecQL4 specific) puromycin-resistant clones were selected. Because complete *RecQL4* silencing dramatically increased the apoptotic cell death, two stable clones (TI339521 vector–transfected clone 6 and TI339524 vector–transfected clone 4; hereafter designated as clone 6 and clone 4) with RecQL4 expression level similar to nontumorigenic RWPE1 cells were selected. These clones showed 80%–90% suppression in RecQL4 protein expression (Fig. 4C). For comparison, clonal cell lines stably expressing empty (Clone 5) and scrambled (Clone 7) vectors were also established.

The increased apoptotic death of *RecQL4*-suppressed cells is presumably due to a high level of spontaneous DNA damage accumulation. To test this possibility, intranuclear distribution of PARP-1, a marker for DNA single-strand breaks and nicks, was monitored in PC3 cells. In comparison to empty vector–transfected cells, *RecQL4*-suppressed clonal cell lines showed increased focalization of PARP-1 protein indicating the spontaneous accumulation of DNA single-strand breaks (Fig. 4D). Additionally, increased focalization of a DNA double-strand break (DSB) marker, 53BP1, as well as specific enrichment of yet another DSB marker, phosphorylated histone H2AX (γ-H2AX), was also detected in *RecQL4*-suppressed clonal cell lines (Fig. 4D). Consistent with DNA strand break accumulation, elevation of structural and numerical chromosomal aberrations were observed in *RecQL4*-suppressed clonal cell lines (Supplementary Fig. S2A). Also, *RecQL4*-suppressed PC3 cells showed elevated apoptotic death after treatment with UVC (Supplementary Fig. S2B) and γ-ray radiation. These findings illustrate that *RecQL4* is critical for protecting the prostate cancer cells from exogenous DNA damage.

Table 1. Summary of immunologic staining analysis of RecQL4 in prostate tumor tissues

Samples	Gleason score	RecQL4 staining intensity			Total
		Low	Medium	High	
Normal		7	1	0	8
BPH		5	2	0	7
PIN		2	2	0	4
Adenocarcinoma	2	1	0	0	1
Adenocarcinoma	3	4	3	0	7
Adenocarcinoma	4	3	4	1	8
Adenocarcinoma	5	3	5	2	10
Adenocarcinoma	6	0	6	4	10
Adenocarcinoma	7	3	13	7	23
Adenocarcinoma	8	0	3	5	8
Adenocarcinoma	9	0	7	12	19
Adenocarcinoma	10	0	3	7	10
Metastatic		0	0	4	4
		28 (23.5%)	49 (41.2%)	42 (35.3%)	119

NOTE: The data obtained from two prostate tumor tissue arrays (PR951 with 48 core samples and PR751 with 75 core samples) were pooled together. Four tumor samples in the arrays without any Gleason score were omitted from the analysis. Out of the four PIN samples, three had Gleason grades I, II, and III, respectively, whereas the fourth sample had Gleason grade of between I and II.

RecQL4 suppression reduces invasiveness and tumorigenicity of prostate cancer cells

To verify whether *RecQL4* silencing affects the tumorigenic potential of PC3 cells, an *in vitro* cell invasion assay was performed. The results showed that cell invasion was greatly reduced in C4 and C6 cell lines (Fig. 5A). *In vivo* tumorigenicity of *RecQL4*-suppressed cell lines was subsequently evaluated in a xenograft mouse model. For this purpose, empty vector (C5) and *RecQL4* shRNA-transfected clonal cell lines (C6 and C4) were injected into athymic nude mice. Representative images of the tumor dissected after 4 weeks from the injected nude mice are shown in Fig. 5B. Tumor size (mm^3) measured over 4 weeks of postinjection is given in Fig. 5C. Tumors grew rapidly in mice injected with PC3 or C5 (empty vector transfected) cells. In contrast, tumor growth was much slower in mice injected with C6 or C4 cells. In fact, one mouse injected with clone 6 remained tumor-free for 4 weeks. These *in vivo* data strongly support the conclusion that *RecQL4* is essential for tumorigenic potential of prostate cancer cells.

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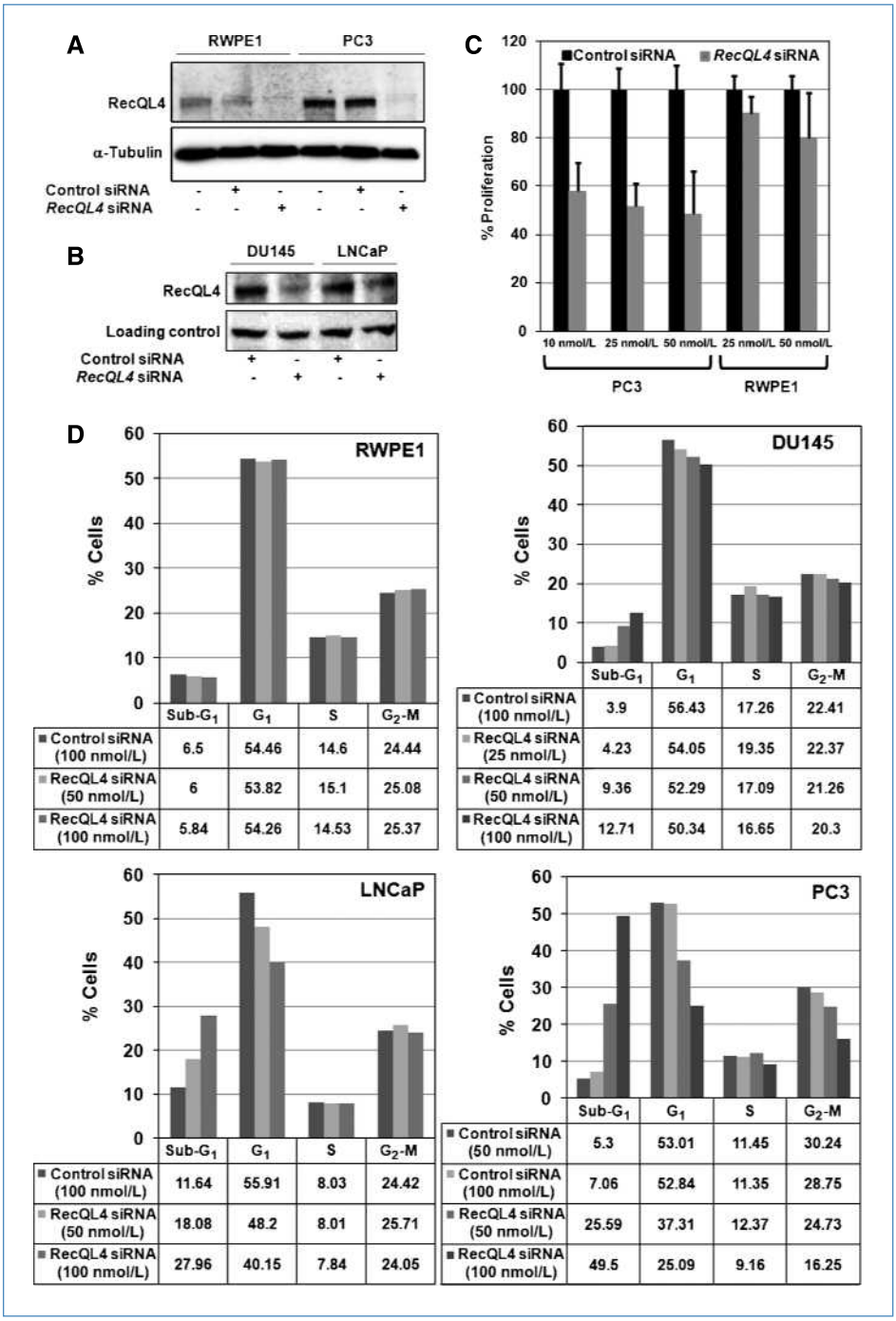

Figure 3. *RecQL4* suppression leads to proliferation failure and apoptosis in metastatic prostate cancer cells. A and B, analysis of *RecQL4* expression in RWPE1 and prostate cancer cell lines 72 h after transfection with control and *RecQL4* specific siRNA. C, effect of *RecQL4* silencing on proliferation by CyQuant assay 48 h after transfection with indicated concentrations of scrambled and *RecQL4*-specific siRNA in RWPE1 and PC3 cell lines. Error bars, SD. D, cell cycle analysis of immortalized RWPE1 and three metastatic prostate cancer cell lines (DU145, LNCaP, and PC3) transfected with 25 to 100 nmol/L of either scrambled control or *RecQL4*-specific siRNA. Cell cycle analysis was performed 72 h after siRNA transfection.

Histone deacetylase inhibitor TSA reduces *RecQL4* expression in prostate cancer cells

As p16 methylation and Rb hyperphosphorylation are frequent molecular events during tumorigenesis, expression levels of p16 and phosphorylated Rb ($Ser^{807/811}$) were monitored in RWPE1 and the three prostate cancer cell lines. Whereas p16 was hardly detectable in DU145, LNCaP, and PC3 cell lines (Fig. 6A), a high level of hyperphosphorylated Rb was observed in the three prostate cancer cell lines. Immunohistochemical studies also showed a positive correlation between Rb hyperphosphorylation and RecQL4 expression in 64 prostate tumor tissues from the PR751 array (Supplementary Fig. S3A).

To reverse p16 methylation and Rb hyperphosphorylation, PC3 cells were treated separately with either a DNA demethylating agent zebularine (200 μmol/L) or a histone deacetylase inhibitor TSA (1 μmol/L) for 48 hours. In both RWPE1 and PC3 cell lines, TSA treatment (1 μmol/L) reduced the proportion of G_1 phase cells with a concomitant increase in G_2-M

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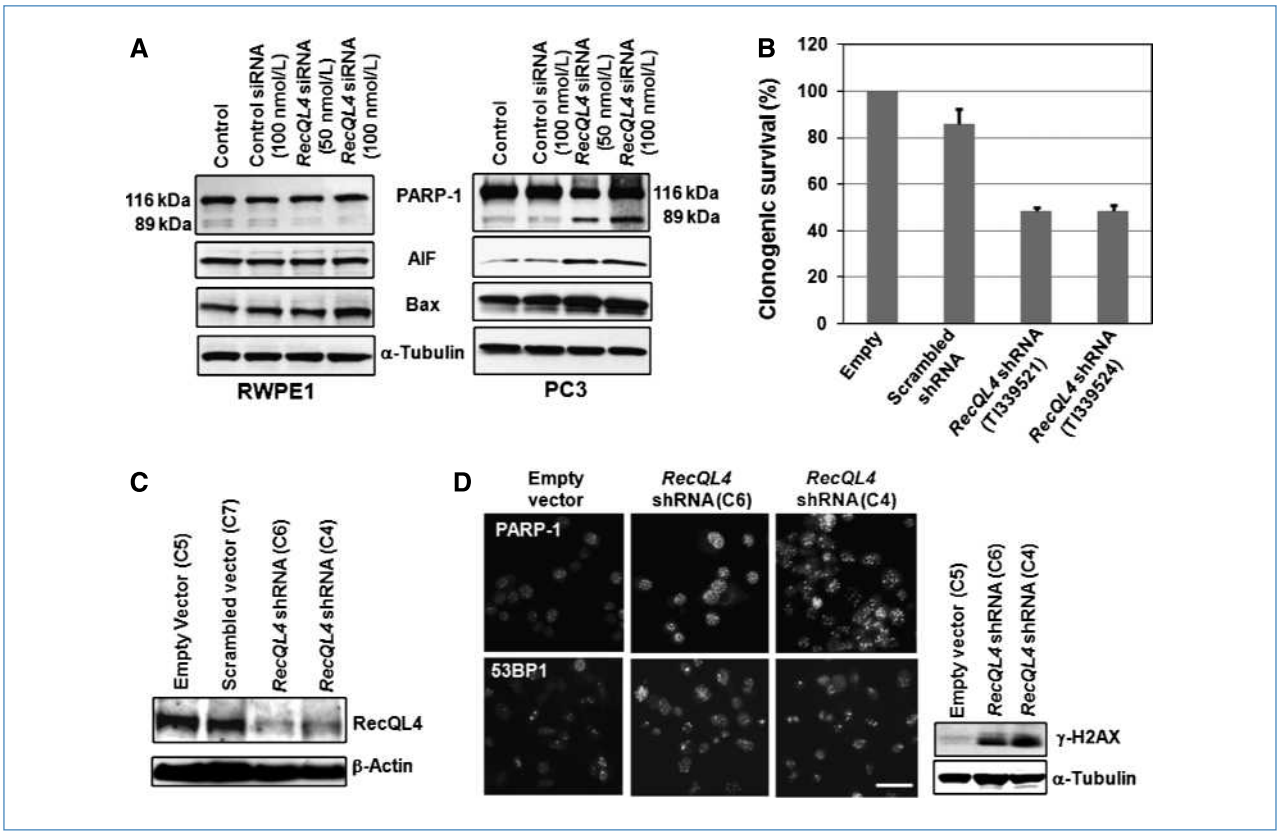

Figure 4. *RecQL4* suppression leads to PARP-1–mediated apoptotic death in prostate cancer cells. A, analysis of cleaved PARP-1, Bax, and AIF proteins in RWPE1 and PC3 cells after 72 h of transfection with control and *RecQL4* siRNA. B, PC3 cells transfected with *RecQL4* shRNA targeting vectors (TI339521 and TI339524) showed reduced survival. C, analysis of RecQL4 expression in empty vector (clone 5), scrambled vector (clone 7), and *RecQL4* shRNA (clones 6 and 4) transfected clonal cell lines of PC3. β-Actin was used as a loading control. D, *RecQL4*-suppressed clonal cell lines (C6 and C4) showed increased focalization of PARP-1 and 53BP1. Western blot analysis showed increased γ-H2AX level in *RecQL4*-suppressed cells. Scale bar, 10 μm.

phase cells but the enrichment of G_2-M phase cells was higher at a lower TSA concentration (0.5 μmol/L). TSA at 1 μmol/L concentration reduced the proportion of S-phase cells only by 2% to 3% relative to mock control in both cell lines (Supplementary Fig. S3B). Strikingly, the apoptotic sub-G_1 cells were more in PC3 cells (23.28%) than in RWPE1 cells (6.42%) after treatment with 1 μmol/L of TSA. Although p16 status remained unaffected, Rb hyperphosphorylation was abolished to a great extent in TSA-treated PC3 cells with a concomitant decline in RecQL4 expression (Fig. 6A). However, RecQL4 expression was reduced by either of the treatments (zebularine and TSA) in RWPE1 cells (Fig. 6A). As cyclin A, cyclin E, and

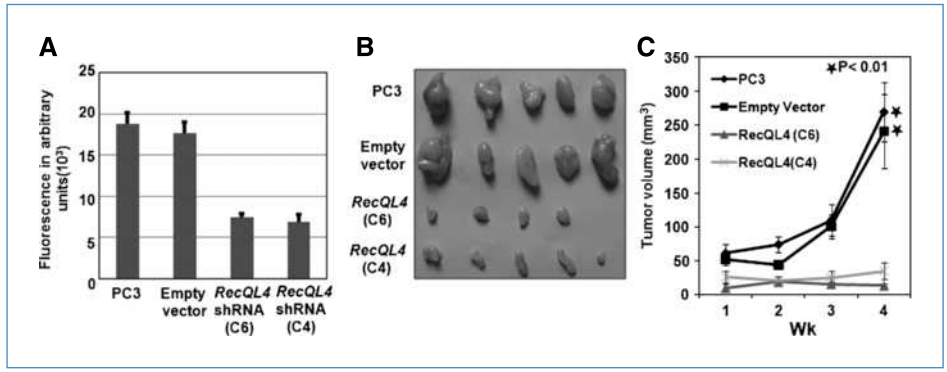

Figure 5. RecQL4 suppression drastically reduces cell invasiveness *in vitro* and tumorigenic growth *in vivo*. A, *RecQL4*-suppressed clonal cell lines (C4 and C6) showed reduced cell invasion capacity. B and C, suppression of *RecQL4* expression in prostate cancer cells reduces tumorigenicity in nude mice. Images of tumors dissected out from the sacrificed mice in B. The tumor size (mm^3) versus days of postinjection in C. Difference in relative tumor volume observed at 4 wk between empty vector and *RecQL4* shRNA-transfected cells was found to be statistically significant. *, $P < 0.01$.

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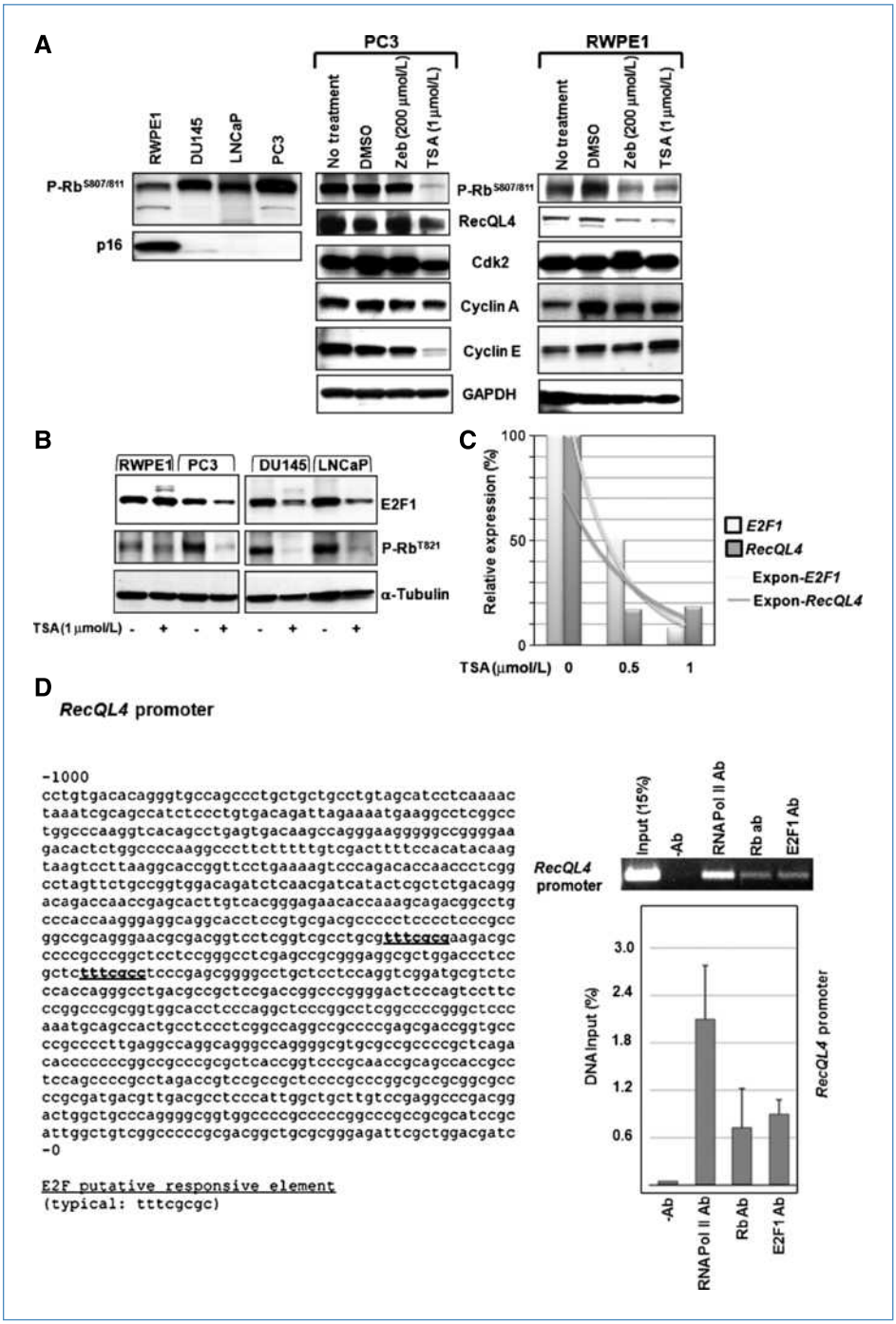

Figure 6. Expression of RecQL4 is modulated by Rb-E2F1 pathway in prostate cancer cells. A, prostate cancer cells showed an inverse correlation between p16 level and Rb hyperphosphorylation ($Ser^{807/811}$). TSA treatment abolished Rb hyperphosphorylation and reduced the expression of RecQL4 in both PC3 and RWPE1 cells. TSA treatment also reduced the levels of cyclin A, cyclin E, and Cdk2 in PC3 cells but not in RWPE1 cells. GAPDH was used as a loading control. B, TSA reduced the levels of E2F1 and phosphorylated Rb (T^{821}) proteins in metastatic prostate cancer cell lines. C, quantitative real-time PCR analysis showing the reduced expression levels of both E2F1 and RecQL4 in DU145 after TSA treatment. D, nucleotide sequence of *RecQL4* promoter highlighting the putative responsive element for E2F1 binding is shown. The ChIP assay was performed in HeLa cells using antibodies specific for Rb and E2F1. Rb- and E2F1-associated *RecQL4* promoter DNA was amplified by PCR using appropriate primers. RNA polymerase II antibody was used as a positive control. Enrichment of the RecQL4 promoter in the immunoprecipitated DNA was quantified by real-time PCR using the primers specific for *RecQL4* promoter. Expon, exponential curve.

Cdk2 mediate Rb phosphorylation, their expression levels were monitored in PC3 and RWPE1 cells (Fig. 6A). The results showed that cyclin A, cyclin E, and Cdk2 levels were noticeably reduced in PC3 cells (Fig. 6A) but were essentially unchanged by TSA treatment in RWPE1 cells. These results suggest that Rb hyperphosphorylation may be regulated by different mechanisms in nontumorigenic and tumorigenic cells. In addition to phospho-Rb $Ser^{807/811}$, expression of phospho-Rb (T^{821}) was also monitored in RWPE1 and the three prostate cancer cell lines. Similar to phospho-Rb ($Ser^{807/811}$), phospho-Rb (T^{821}) expression was also reduced in all the cell lines examined (Fig. 6B).

RT-PCR analysis showed that TSA treatment for 48 hours caused a dose-dependent reduction in *RecQL4* mRNA in DU145, LNCaP, and PC3 cell lines (Supplementary Fig. S3C). We next verified whether the reduced RecQL4

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expression after TSA treatment is due to reduced E2F1 expression. We found that E2F1 protein expression was considerably reduced only in TSA-treated prostate cancer cell lines but not in RWPE1 cells (Fig. 6B). Also, TSA reduced the expression of both *RecQL4* and *E2F1* mRNAs in a dose-dependent manner in DU145 cells (Fig. 6C) as well as in LNCaP and PC3 cell lines (data not shown). Collectively, these studies suggest that elevation of *RecQL4* expression in prostate cancer cells and prostate tumor tissues is likely due to deregulation of Rb-E2F1 pathway.

Rb and E2F1 proteins physically interact with *RecQL4* promoter *in vivo*

The nucleotide sequence of the *RecQL4* promoter with potential target sequence for E2F1 binding is shown in Fig. 6D. To test whether Rb and E2F1 proteins directly regulate transcription of *RecQL4* via these sites, ChIP assay was performed to detect E2F1 and Rb binding to the *RecQL4* promoter in HeLa cells (Fig. 6D). RT-PCR analysis of immunoprecipitated HeLa chromatin DNA detected the *RecQL4* promoter bound to Rb and E2F1 proteins (Fig. 6D). The *RecQL4* promoter was undetectable in the negative control without any antibody, whereas a positive control using RNA polymerase II antibody detected a specific enrichment of the *RecQL4* promoter. Quantification of the *RecQL4* promoter bound to Rb and E2F1 proteins was analyzed by real-time PCR (Fig. 6D). These results suggest the possibility that Rb and E2F1 regulate *RecQL4* expression in prostate cancer cells.

Discussion

Here, we report the novel finding that RecQL4 helicase, which maps to 8q24.3, is upregulated in prostate cancer cells and tissues. The salient findings of this study are (*a*) *RecQL4* expression is elevated in metastatic prostate cancer cells and tumor tissues, (*b*) *RecQL4* depletion causes proliferation failure and apoptosis in prostate cancer cells lines, (*c*) *RecQL4* protects the genomic integrity of prostate cancer cells from endogenous and exogenous DNA damage, and (*d*) *RecQL4* suppression in metastatic PC3 cells drastically reduced their tumorigenic potential both *in vitro* and *in vivo*. These data support our hypothesis that *RecQL4* is a critical factor for prostate carcinogenesis. Based on our study, we propose that elevated *RecQL4* expression confers survival advantage to prostate cancer cells by protecting their genomic integrity from endogenous and exogenous DNA damage. In support, several earlier studies have shown a direct or indirect participation of RecQL4 in diverse DNA repair pathways (10, 32–36). Collectively, our findings show that RecQL4 is a critical factor for prostate cancer cell growth and viability.

Unlimited replicative potential is a hallmark of immortalized and metastatic prostate cancer cells. Recent lines of evidence indicate an essential role for RecQL4 in DNA replication initiation (11, 13, 40–42). Furthermore, the NH_2 terminal domain of RecQL4 shares extensive homology to an essential DNA replication protein, Sld2 (40). Recent studies have established a direct interaction of RecQL4 with factors involved in replisome assembly (43, 44). A direct interaction of RecQL4 with minimal chromosome maintenance complex (MCM 10 and MCM 2–7 helicase), CDC45, and GINS was recently documented (44). A more direct role of RecQL1 and RecQL4 in DNA replication initiation was also recently established (45). Therefore, reduced proliferation observed in *RecQL4*-suppressed prostate cancer cells is probably due to deficiencies in DNA replication initiation.

Liu and colleagues (37) showed that disruption of the Rb and E2F pathway leads to the enhanced expression of RecQ helicases using the mouse model systems deficient in these pathways. The present study shows that Rb hyperphosphorylation and RecQL4 expression correlated with each other in both prostate cancer cells and tissues. Consistent with this, TSA treatment resulted in the abolition of Rb hyperphosphorylation and reduced expression of both E2F1 and RecQL4. Loss of Rb hyperphosphorylation was accompanied by the reduction in the levels of Cdk2, cyclin A, and cyclin E in PC3 cells. Collectively, these data suggest that *RecQL4* expression may be regulated by the Rb-E2F1 pathway in prostate cancer cells. Other mechanisms may also regulate *RecQL4* expression in prostate cancer cells. For example, p53-mediated transcriptional repression of *RecQL4* has been documented in the literature (38) in immortalized human fibroblasts by wild-type p53 but not by tumor-derived mutant p53 forms. However, in this study, elevated *RecQL4* expression was found both in p53 mutated (DU145 and PC3) and p53 wild-type (LNCaP) prostate cancer cell lines (39). Thus, different mechanisms may regulate *RecQL4* expression in immortalized fibroblasts and metastatic prostate cancer cells.

Among the five RecQ helicases, RecQL4 alone showed an elevated expression consistently in all the six prostate cancer cells lines examined by us. In contrast, BLM helicase was highly expressed only in DU145 cells, whereas the expression levels of other human RecQ helicases (RecQL1, RecQL5, and WRN) were grossly similar in immortalized prostate epithelial and prostate cancer cell lines (Supplementary Fig. S4). On this basis, we propose that elevated *RecQL4* expression has two well-defined biological functions in prostate carcinogenesis (*a*) to confer infinite proliferation potential to prostate cancer cells through its direct participation in DNA replication and (*b*) to protect the genomic integrity of prostate cancer cells from endogenous and exogenous DNA damage by regulating the efficiency of diverse DNA repair pathways. Observations of the reduced proliferation, clonogenic survival, cell invasion, and *in vivo* tumorigenicity of *RecQL4*-suppressed metastatic prostate cancer cells convincingly favor the aforementioned functions of *RecQL4* in prostate cancer cells. Specific elevation of *RecQL4* expression in the metastatic prostate cancer cells and tumor tissues indicate that *RecQL4* may be a novel biomarker for the advanced stage of prostate cancer. Additionally, *RecQL4* suppression by TSA raises a potential possibility for development of new

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therapeutic strategies for *RecQL4* targeting in prostate cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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