The Retinoblastoma Tumor Suppressor Modulates DNA Repair and Radioresponsiveness

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Abstract

Purpose: Perturbations in the retinoblastoma pathway are over-represented in advanced prostate cancer; retinoblastoma loss promotes bypass of first-line hormone therapy. Conversely, preliminary studies suggested that retinoblastoma-deficient tumors may become sensitized to a subset of DNA-damaging agents. Here, the molecular and in vivo consequence of retinoblastoma status was analyzed in models of clinical relevance.

Experimental Design: Experimental work was performed with multiple isogenic prostate cancer cell lines (hormone sensitive: LNCaP and LAPC4 cells and hormone resistant C42, 22Rv1 cells; stable knockdown of retinoblastoma using shRNA). Multiple mechanisms were interrogated including cell cycle, apoptosis, and DNA damage repair. Transcriptome analysis was performed, validated, and mechanisms discerned. Cell survival was measured using clonogenic cell survival assay and in vivo analysis was performed in nude mice with human derived tumor xenografts.

Results: Loss of retinoblastoma enhanced the radioresponsiveness of both hormone-sensitive and castrate-resistant prostate cancer. Hypersensitivity to ionizing radiation was not mediated by cell cycle or p53. Retinoblastoma loss led to alteration in DNA damage repair and activation of the NF-kB pathway and subsequent cellular apoptosis through PLK3. In vivo xenografts of retinoblastoma-deficient tumors exhibited diminished tumor mass, lower PSA kinetics, and decreased tumor growth after treatment with ionizing radiation (P < 0.05).

Conclusions: Loss of retinoblastoma confers increased radiosensitivity in prostate cancer. This hypersensitization was mediated by alterations in apoptotic signaling. Combined, these not only provide insight into the molecular consequence of retinoblastoma loss, but also credential retinoblastoma status as a putative biomarker for predicting response to radiotherapy. Clin Cancer Res; 20(21); 5468–82. ©2014 AACR.

Introduction

The retinoblastoma protein (RB1) is a tumor suppressor protein and is functionally inactivated in several major cancers (1). Retinoblastoma belongs to the pocket protein family (pRb, p107, and p130), whose members have a pocket for the functional binding of other proteins and while present throughout the cell cycle, its function is regulated in a cell-cycle-dependent manner (2). In quiescent cells, retinoblastoma is hypophosphorylated and forms a repressive transcriptional complex on E2F-regulated gene promoters to inhibit cell cycle. However, in response to mitogenic signals, retinoblastoma phosphorylation disrupts the retinoblastoma–E2F interactions facilitating G1–S cell-cycle progression.

Retinoblastoma is a key regulator of multiple cellular functions including cell proliferation, apoptosis, differentiation, genome integrity, quiescence, senescence, and DNA repair (3–6). Retinoblastoma function is altered in several tumor types through distinct mechanisms, which are often tissue specific. Within prostate cancer, RB1 loss is deregulated in approximately 5% of primary tumors, and up to
apoptosis via NF-κB–mediated cellular apoptosis through polo-like kinase 3 (PLK3) modulation. PLK3 is a cytokine inducible kinase and has been shown to function as potent inducer of apoptosis via NF-κB binding to the PLK3 promoter (12). In addition, the results are recapitulated using human xenografts. Together, these in vitro and in vivo data reveal a new paradigm for the role of retinoblastoma in regulating cell survival in prostate cancer after treatment with radiotherapy and reveal the potential to personalize therapy in prostate cancer patients based on retinoblastoma status.

Materials and Methods

Cell culture

LNCaP and C4-2 cells were maintained in improved minimum essential medium supplemented with 5% FBS (heat-inactivated FBS). LAPC4 cells were maintained in Iscove modified Dulbecco medium supplemented with 10% FBS. 22Rv1 cells were maintained in RPMI supplemented with 10% FBS (Atlanta Biologicals). For steroid-depleted conditions, cells were plated in appropriate phenol red–free media supplemented with 5% to 10% CDT (GE Healthcare Life Sciences, Hyclone Laboratories).

Immunofluorescence analysis

Immunofluorescence staining was performed as previously described (10). Immunolocalization of γH2AX, 53BP1, cleaved caspase-3, and NFκBp50 was carried out by using a confocal microscopy (Nikon, Core Facility at Thomas Jefferson University, Philadelphia, PA).

Cell growth assay

Retinoblastoma-proficient and -deficient LNCaP, LAPC4, C4-2, and 22Rv1 cells were seeded at equal densities (1 × 10^5), exposed to IR (PanTakOrthovoltage X-ray irradiator, calibrated daily using a Victoreen dosimeter), and harvested at indicated time points. At the time of harvest, cell number was determined using Trypan blue exclusion dye by using a hemocytometer. Cells were seeded at the above densities and transfected and infected with PLK3 cDNA (Addgene) or adenovirus harboring IκBα DN (SA mutation; Vector Biolabs).

RNA isolation and microarray analysis

Actively growing retinoblastoma-proficient and retinoblastoma-deficient LNCaP cells were exposed to IR (10 Gy) and the cells were harvested 24 hours after IR administration (three independent biologic replicates). Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies). Microarray was carried out as described (13). A 1.5-fold differentially expressed gene list was generated. The differentially expressed gene list was loaded into Ingenuity Pathway Analysis (IPA) 8.0 software (http://www.ingenuity.com) to perform biologic network and functional analyses and genes were highlighted. Microarray data was deposited in NCBI/GEO website and the GEO accession number GSE58711. \(P < 0.05\) was considered as statistically significant. Microarray validation was conducted using quantitative real-time (qRT)-PCR and quantified using a \(\Delta\Delta C_t\) method.

Flow cytometry analysis

Cell proliferation was assessed by bromodeoxyuridine (BrdUrd) incorporation using flow cytometry as previously described (7). Loss of retinoblastoma function, most commonly via allelic loss, facilitates development of resistance to hormone ablative therapies (8). Retinoblastoma status has also been shown to alter response to genotoxic insults (9). Despite challenge with various chemotherapeutics, retinoblastoma-deficient MEFs failed to halt cell-cycle progression resulting in incorrect DNA repair and cell death. However, within the context of prostate cancer cells, retinoblastoma loss failed to alter cell growth despite challenge with an HDAC inhibitor and, surprisingly, lead to resistance with cisplatin exposure in vitro (10). Moreover, treatment with antimicrotubule agents and a topoisomerase inhibitor yielded increased sensitivity in the retinoblastoma-depleted cells suggesting that cellular response to therapeutic intervention in prostate cancer cells is agent specific. Radiotherapy is a well-established treatment modality for localized and locally advanced prostate cancer. However, the role of radiotherapy has expanded with the introduction of radium-223 (11), which has yielded an improvement in survival in men with metastatic castrate-resistant prostate cancer. Despite the high frequency of retinoblastoma inactivation, few studies have addressed the impact of this event on cellular response to ionizing radiation (IR). Herein, we delineated the impact of retinoblastoma function on response to IR using a panel of human isogenic prostate cancer lines with stable knockdown of retinoblastoma. In this study, we show for the first time that loss of retinoblastoma function results in increased radiosensitization of human prostate cancer cells, using both short-term growth as well as clonogenic survival assays. Furthermore, the increased sensitivity is mediated through alterations in both apoptotic as well as DNA damage and repair pathways. Furthermore, the study identified a key mechanism of NF-kB–mediated cellular apoptosis through polo-like kinase 3 (PLK3) modulation. PLK3 is a cytokine inducible kinase and has been shown to function as potent inducer of apoptosis via NF-kB binding to the PLK3 promoter (12). In

Translational Relevance

Loss-of-function of the retinoblastoma tumor suppressor protein in prostate cancer yields a castrate-resistant phenotype. Given the survival benefit of radiotherapy in the management of men with advanced prostate cancer, one outstanding issue is the impact of retinoblastoma function on sensitization to DNA-damaging agents. In this study, we show that retinoblastoma loss promotes sensitization to genotoxic stress through mechanisms distinct from cell-cycle checkpoint control and identify retinoblastoma as a potent effector of the response to radiotherapy. The findings of this study suggest that future radiation trials should interrogate retinoblastoma status as a potential biomarker of therapy response.

30% to 40% in metastatic or castration-resistant prostate cancer (CRPC) samples (7). Loss of retinoblastoma function, most commonly via allelic loss, facilitates development of resistance to hormone ablative therapies (8).
described (14). Flow cytometry analysis was performed (GE Healthcare) and the data were analyzed by using FlowJo version 8.8 software.

**Clonogenic assay**

Clonogenic survival assays were carried out as previously described (15). Only colonies of 50 or more cells were counted. Three replicates per dose were studied. Survival curves were generated. The surviving fraction value was corrected for cellular multiplicity to provide single-cell survival.

**Western blotting analysis**

For protein analysis, cells were harvested by trypsinization and cell lysis was performed. The membranes were immunoblotted for pRB (BD Biosciences), actin, LaminB, p21, NFXB p50, NFXB p65, 1k8t cleaved caspase-3 (Santa Cruz Biotechnology Inc and Abcam), PLK3 (Sigma-Aldrich), DNA ligase IV (Abcam), CDC25A, CDK2, and p53 (Santa Cruz Biotechnology Inc) by standard techniques and visualized using enhanced Western Lightening Chemiluminescence (Perkin-Elmer Life Sciences).

**Chromatin immunoprecipitation assay**

Following IR, as described above, LNCaP and LAPC4 cells were cross-linked with formaldehyde and processed for chromatin immunoprecipitation (ChIP) analysis as previously described (8). Equal concentrations of chromatin from all treatment groups were preclared with protein Agarose beads in the presence of BSA to reduce nonspecific background. After removal of beads by centrifugation, 2 μg of NFXBp50 or NFXBp65 antibodies (Santa Cruz Biotechnology) were added and kept at 4°C for overnight on a rotary platform. The immunoprecipitated DNA was purified using PCR purification kit (Qiagen) and resuspended in 50 μL of sterile water. The purified DNAs and input DNA were analyzed by semi quantitative PCR using PLK3 promoter targeting by using a forward 5’-GCC CTT GTC TAG CAT TTG AG-3’ and a reverse primer 5’-CCA TCA CAC CCG CCTAATT-3' sequences, as described in refs. (8, 12). Input DNA served as a positive control, whereas rabbit IgG and a non-E2F/retinoblastoma target albumin promoter served as negative controls. PCR products were resolved on agarose gel and the images were captured using Bio-Rad Hemidoc Imager (Bio-Rad Laboratories Inc), the band intensities were measured using ImageJ, and the results were presented graphically using GraphPad prism version 6 (GraphPad Software, Inc.). The quantitative PCR results were quantitated using the ΔΔCt method.

**Retinoblastoma knockdown**

Retinoblastoma knockdown was carried out as described in ref. (8). Control and knockdown LNCaP, LAPC4, 22RV1, and C4-2 cells were generated by transfection with either shRNA plasmid directed against retinoblastoma (MSCV-Rb3C; targeted sequence: 5’-CGCACTAATCGGT-TAGAATGTTAGACA-3’) or a control plasmid (MSCV donor) using Lipofectin Transfection Reagent (Invitrogen, Life Technologies). Retrovirus encoding shRb plasmid (MSCV-LMP Rb88; targeted sequence: 5-GAAAGGCATGT-GAACCTA-3) or control plasmid (MSCV donor) were used to create retinoblastoma knockdown or control LAPC-4 stable clones. After selection with puromycin for 6 to 7 days, stable clones were isolated and characterized. Puromycin-selected clones were subjected to retinoblastoma mRNA (qRT-PCR) and retinoblastoma protein analysis (retinoblastoma immunoblotting) and selected retinoblastoma-deficient clones were further utilized for the study.

**Comet assay**

Alkaline comet assay was performed as described in ref. (16) using the Trevigen comet assay kit (Trevigen Inc). Slides were visualized using epifluorescence microscopy. The images were analyzed using CometScoring software (TriTek Corp).

**Transcription factor ELISA**

Transcription factor ELISA (TF ELISA) were performed as previously described (17). The protein–DNA complex was detected by chromogenic substrate. Absorbance of the samples was measured at 450 nm using microplate reader (Bio-Tek I Spectrophotometer instruments).

**Xenografts and IR treatments**

Xenografts were generated as described previously (8). Retinoblastoma-proficient and -deficient LNCaP cells (4 × 10⁶) were individually mixed (1:1) with Matrigel in a 200 μL volume (BD Biosciences) and the cells were implanted subcutaneously into the flanks of NCR/nu/nu (athymic) male mice. Once the tumors reached 150 mm³ volume, retinoblastoma-proficient and retinoblastoma-deficient tumors were exposed to IR (10 Gy, PanTak Orthovoltage X-ray irradiator). Tumor volumes were measured weekly with calipers, serum prostate-specific antigen (PSA) levels were determined, and PSA doubling times were calculated. In shRb xenografts, maintenance of retinoblastoma knockdown in vivo was verified by qRT-PCR analysis of the human Rb1 transcript as described in ref. (8, 18). Retinoblastoma transcript levels as measured by qRT-PCR ranged from 10% to 90%. Maintenance of retinoblastoma silencing was defined as having 30% or less of Rb1 transcript level when compared with wild-type xenograft tumors. Thirty percent of tumors failed to maintain loss of retinoblastoma transcript and were not included in the analysis given the uncertainty of the timing of retinoblastoma function. Animal studies were conducted in accordance with the principles and procedures outlined by the NIH guidelines and the Institutional Animal Care and Use Committee of Thomas Jefferson University (Philadelphia, PA).

**PLK3 ectopic expression and knockdown**

PLK3 cDNA transfection and knockdown was carried as described in the manufacturer’s protocol. Briefly, 10 μg of PLK3 cDNA (Addgene) or control vector (PCDNA3) or PLK3 shRNA (Applied Biological Materials, Inc.) or shRNA
vector alone were transiently transfected in LNCaP shCon or LNCaP shRB or LAPC4 shCon or LAPC4 shRB cells with Lipofectamine 2000 (Invitrogen, Life Technologies). The PLK3 overexpression or PLK3 knockdown was confirmed by PLK3 immunoblotting. The cells expressing PLK3 or PLK3-deficient cells were used for generating growth curve with radiation or no radiation. Cells were also analyzed for cleaved caspase-3 immunoblotting.

**Ectopic expression of dominant-negative IxBosA**

shRB cells (LNCaP and LAPC4) were infected with adenovirus harboring dominant-negative IxBosA (Vector Biolabs) and exposed to IR 10 Gy, processed for cell growth assay and cleaved caspase-3 apoptotic marker immunofluorescence, and Western blotting analysis.

**Retinoblastoma immunohistochemical analyses**

Retinoblastoma immunohistochemistry was performed as described in ref. (8, 19). Eleven patients were identified in our institution that had biopsy-proven local recurrence following primary radiotherapy (5 patients received external beam radiotherapy and received brachytherapy). Biopsy specimens or whole mount glands were used for immunohistochemical analyses. Five micrometer sections were deparaffinized, antigen retrieval was performed in 10 mmol/L EDTA (pH 9) for 10 minutes in a pressure cooker, and slides were incubated with 3% H2O2 for 10 minutes and then blocked with avidin/biotin blocking solution (Vector Biolabs) for 30 minutes and incubated in a 5% chicken/goat/horse serum solution for 2 hours. Sections were incubated with anti-retinoblastoma antibody overnight at 4°C ([4H1] Mouse mAb #9309, Cell Signaling Technology, Inc.; concentration 1:200). Negative control slides were incubated with mouse anti-MOPC21 (generated from a hybridoma obtained from ATCC) at the same concentration as the primary antibody. Slides were then incubated with horse anti-mouse biotinylated secondary antibody (1:150, Vector Biolabs) for 30 minutes, developed using Vectastain ABC (Vector Biolabs) and stable DAB (Invitrogen, Life Technologies) counterstained with hematoxylin, dehydrated, and mounted with Cytoseal XYL (Richard Allan Scientific). The stain was interpreted as the percentage of tumor cells with nuclear staining and intensity of staining as 3+ (strong staining similar to positive control), 2+ (moderate staining), 1+ (weak staining), and 0 (no staining). Photomicrographs were taken by using bright field microscope (20×) and the scoring was performed by a clinical pathologist (an experienced staff pathologist; Thomas Jefferson University Hospital, Philadelphia, PA).

**Analysis of PLK3 and RB1 correlation in prostate cancer**

A normalized mRNA expression dataset of prostate adenocarcinoma (7) was downloaded from the cBioPortal for cancer genomics (http://www.cbioportal.org/public-portal/) and was used to evaluate coexpression of RB1 and PLK3 transcript levels. This dataset includes mRNA profiles for 29 normal prostate, 131 primary tumor, and 19 metastasis samples. Pearson correlation coefficient was calculated within normal prostate and prostate cancer groups. RB1 transcript levels were used to infer prostate cancer sample retinoblastoma status, where retinoblastoma-deficient samples were defined below the median RB1 expression and retinoblastoma-proficient samples were defined above the median. A two-tailed \( t \) test for populations of unequal variance was used to evaluate the significance of differential PLK3 expression in retinoblastoma-proficient versus normal and retinoblastoma-deficient versus retinoblastoma-proficient groups.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 6.0) software (GraphPadPrism Software, Inc.). All the data were analyzed for statistical significance using Student test/one-way ANOVA. For all experiments, \( P < 0.05 \) was considered statistically significant.

**Results**

**Retinoblastoma status dictates the cellular response to radiation in both early- and late-stage cancers**

The retinoblastoma tumor suppressor protein (pRB) pathway is a key regulator of cell cycle in coordination with E2Fs. Retinoblastoma inactivation occurs during prostate cancer progression and has been correlated with poor outcome (8). In human samples, retinoblastoma loss-of-function occurs commonly via loss of heterozygosity (20). To delineate the role of retinoblastoma in modulating the response to radiotherapy, clinically relevant hormone-sensitive and castrate-resistant human isogenic prostate cancer cell lines were utilized that expressed either shRB or control as previously described (8). Retinoblastoma knockdown was verified using protein analysis across multiple prostate cancer cell lines (Fig. 1A and B, left). First, in vitro clonogenic assays were performed. In the presence of androgen, retinoblastoma-deficient LNCaP and LAPC4 cells showed increased radiosensitivity across all radiation doses studied, as compared with the isogenic retinoblastoma-proficient pairs. Statistical significance was observed after both 8 and 10 Gy exposure, indicating conservation of this result across multiple ranges of DNA damage (Fig. 1C). Short-term growth assays using 10 Gy of IR confirmed these findings, as control LNCaP and LAPC4 demonstrated greater viable cell numbers than their isogenic pairs (Fig. 1A) in both hormone-enriched and hormone-free media. Given that retinoblastoma knockdown had been demonstrated to result in a growth advantage in a hormone-free environment (10), short-term growth assays were performed with isogenic pairs (shRB and shControl) derived from castrate-resistant prostate cancer–deficient C4-2 and 22Rv1 cell lines (Fig. 1B) in both hormone-competent and hormone-free conditions. Together, these results suggest that retinoblastoma loss sensitizes prostate cancer to IR, irrespective of hormone microenvironment. Given the prominent role of cell cycle in the manifestation of DNA damage, the impact of cell-cycle checkpoints was investigated.
Figure 1. Retinoblastoma (RB) status dictates the cellular response to radiation in both hormone-sensitive and castrate-resistant cancers. Actively growing LNCaP, LAPC4, C4-2, and 22Rv1 cells were exposed to radiotherapy and processed for further analysis. A, retinoblastoma Western blotting analysis in hormone-responsive LNCaP and LAPC4 cells and actin loading control (top left). Cell number analysis of hormone-responsive LNCaP and LAPC4 cells in hormone-enriched and hormone-free media in response to radiotherapy (10 Gy; top right). B, retinoblastoma Western blotting analysis in castrate-resistant C4-2 and 22Rv1 cells and actin loading control (bottom left). Cell number analysis of C4-2 and 22Rv1 cells in hormone-enriched and hormone-free media in response to radiotherapy (10 Gy; bottom right). C, clonogenic assay in LNCaP and LAPC4 cells. Each data point is a mean ± SD from three or more independent experiments. **, *P < 0.05 were considered as statistically significant.
Figure 2. Retinoblastoma (RB) status alters the transcriptional response to DNA damage. Actively growing LNCaP and LAPC4 cells were exposed to radiotherapy and processed for further analysis. A, microarray analysis (in silico) generated heatmap of 1,131 differentially regulated genes in retinoblastoma-proficient and deficient LNCaP cells 24 hours post-IR (10 Gy). B, IPA software generated deregulated functional pathways in retinoblastoma-proficient and deficient LNCaP cells in response to radiotherapy. C, qRT-PCR validation of functionally important genes from the microarray data. Each data point is a mean ± SD from three or more independent experiments. **, P < 0.05 were considered as statistically significant.
Figure 3. Retinoblastoma (RB) loss alters DNA damage and repair capacity in response to radiation. Actively growing LNCaP and LAPC4 cells were exposed to radiotherapy and processed for further analysis. A, in silico analysis generated heatmap of DNA damage and repair pathway genes from retinoblastoma-proficient and deficient LNCaP cells after 24 hours post-IR (10 Gy). (Continued on the following page.)
The impact of retinoblastoma on the radiation response is independent of p53

DNA damage induces cell-intrinsic checkpoints, including p21, p53, and retinoblastoma (21). Further prior reports demonstrated that p53 and retinoblastoma can exert partially overlapping roles in the setting of inactivation of either tumor suppressor (22). Thus, complementary upregulation of p21 or p53 may occur in the setting of retinoblastoma knockdown after exposure to IR. In prostate cancer models, upregulation and stabilization of p53 has been correlated with the induction of apoptosis (23). However, no difference in either protein induction or stabilization was observed for either p21 or p53 after exposure to DNA damage (Supplementary Fig. S1A and S1B) in either LNCaP or LARC4 regardless of retinoblastoma status. These findings indicate that cell proliferation and cell-cycle control may be altered in retinoblastoma knockdown cells given the lack of compensation for other prominent checkpoint regulators.

Retinoblastoma status does not impinge on the alterations of cell cycle after radiation

Given the prominent role of retinoblastoma as a G1 cell-cycle checkpoint through the regulation of E2 family transcription factors, it was hypothesized that retinoblastoma knockdown would alter cell-cycle regulation after radiation. Furthermore, in multiple cell lines with disrupted retinoblastoma function, it has been consistently shown that retinoblastoma deficiency allows cells to efficiently bypass the cell-cycle inhibitory response to DNA-damaging chemotherapeutic agents such as cisplatin (9). In both retinoblastoma-proficient and -deficient prostate cancer cells, exposure to IR resulted in a G1 cell-cycle arrest. However, it was surprising that no differential response in cell-cycle progression as measured by BrdUrd incorporation was noted after exposure to IR regardless of retinoblastoma status (Supplementary Fig. S1C–S1F). No differences were observed in CDC25A expression regardless of retinoblastoma status with or without exposure to IR (Supplementary Fig. S1G). Upregulation of CDK2 expression occurred in retinoblastoma-deficient cells, but was not altered by exposure to IR (Supplementary Fig. S1G). Given that alterations in cell survival do not appear to be accounted through alterations in cell-cycle proliferation or cell-cycle checkpoint response, other mechanisms were explored, which could putatively impinge on cell survival after genotoxic insult.

Retinoblastoma status alters the transcriptional response to DNA damage

Given that cell survival is a complex process and regulated by multiple underlying mechanisms, retinoblastoma-proficient and -deficient LNCaP cells were exposed to IR and followed by mRNA microarray analysis. After background correction and normalization, a gene list consisting of 1,131 differentially (at least 1.5-fold change in expression) regulated genes was observed (Fig. 2A). Using biologic network and functional analysis, gene ontology demonstrated that 11 important functional pathways were significantly altered (Fig. 2B) including cell death and survival as well as DNA replication, recombination, and repair, as a result of retinoblastoma knockdown in the setting of DNA damage. An extended qRT-PCR validation was performed (Fig. 2C), confirming the findings of the microarray. Together, these data suggest that cell survival may be regulated through alterations of DNA damage/repair and increased apoptosis in retinoblastoma-deficient cells.

Retinoblastoma loss alters DNA damage repair pathway

Microarray transcript analysis demonstrated that DNA damage and repair pathway genes were elevated in retinoblastoma-deficient LNCaP cells (Fig. 3A). IR results in multiple DNA aberrations including base excision as well as double-strand DNA breaks (24), and microarray validation reveals that multiple base excision repair genes including POLB, POLK, and REV3L (Fig. 3B) as well as the DNA-damage-induced meiotic recombination gene SYCP3 (25) were upregulated and alternatively DNA ligase IV was downregulated (Fig. 3C) in shRB cells in the setting of radiation-induced damage (5). DNA ligase IV is an ATP-dependent DNA ligase that joins double-strand breaks during the nonhomologous endjoining pathway of double-strand break repair (26). These findings suggest that retinoblastoma may impact the DNA repair machinery. Given that SYCP3, γH2AX, and 53BP1 are involved in DNA repair via similar mechanisms, we assessed the ability to repair DNA double-strand breaks (27). γH2AX and 53BP1 foci were significantly elevated in shRB LNCaP cells as compared with control cells, indicating that radiation-induced DNA damage depends on the retinoblastoma status of the cells (Fig. 3D, left with quantification on right panel). The results were recapitulated in LARC4 (Fig. 3E); shRB cells showed markedly diminished capacity to repair double-strand breaks up to 24 hours after treatment. Comet assay recapitulated these findings demonstrating that retinoblastoma deficiency radiosensitizes prostate cancer cells as shown with longer tail moments indicating higher amounts of DNA breaks (Fig. 3F). Taken together, markers of DNA damage support the hypothesis that retinoblastoma loss alters DNA repair mechanisms. Thus, these data reveal for the first
time that retinoblastoma promotes DNA double-strand break repair independent of the ability of retinoblastoma to regulate cell-cycle progression.

**Retinoblastoma deficiency leads to activation of NF-κB pathway and cellular apoptosis in response to IR**

Retinoblastoma has been shown to function as an anti-apoptotic factor (28) and microarray analysis demonstrated induction of proapoptotic transcripts in the setting of retinoblastoma depletion (Supplementary Fig. S2). Many of the altered gene transcripts are proapoptotic genes regulated via NF-κB including DR4, TRAIL, RIP1 Fas, Fasl, MYC, NOTCH1, and PLK3 (29). The behavior of the transcription factor NF-κB as a promoter or antagonist of apoptosis depends on the apoptotic stimulus. Higher total levels as well as increased nuclear translocation of NF-κB p50 and p65 were observed in retinoblastoma-deficient cells in response to radiation (Fig. 4A). Further immunolocalization and TF ELISA analysis reveal that nuclear and DNA-bound NF-κB p50 was elevated in retinoblastoma-deficient LNCaP and LAPC4 cells (Fig. 4B and C). To mimic NF-κB knockdown, we exogenously expressed a dominant-negative IκBα (SA), which retains NF-κB in the cytoplasm, thus preventing NF-κB–mediated downstream signaling. This modulation decreased nuclear translocation (Fig. 4D, Western blots) and decreased the radiosensitivity of shRB LNCaP and LAPC4 cells (Fig. 4D, growth curves). TF ELISA analysis reveals that nuclear and DNA-bound NF-κB p50 was diminished in retinoblastoma-deficient LNCaP and LAPC4 cells with the introduction of the dominant-negative IκBα (Supplementary Fig. S3A and S3B). Elevated levels of NF-κB p50 and p65 protein have been demonstrated to activate the apoptotic pathway (29). Knockdown of retinoblastoma resulted in increased apoptosis in the setting of IR (Fig. 4E) and inhibition of NF-κB via IκBα DN diminished cleaved caspase-3 levels (Fig. 4F). Thus, retinoblastoma loss results in upregulation and increased nuclear translocation of NF-κB with subsequent induction of apoptosis in the setting of IR.

**Modulation of PLK3 alters cellular apoptosis through an NF-κB–dependent manner**

NF-κB proapoptotic signaling has been demonstrated to be mediated via direct binding to the polo-kinase 3 promoter (12). This activation of PLK3 resulted in induction of apoptosis. ChIP assay and qRT-PCR revealed higher levels of NF-κB p50 and p65 bound to the PLK3 promoter (Fig. 5A). These increased bindings correlated with increased expression of the PLK3 transcript in the setting of retinoblastoma knockdown (Fig. 5B). Furthermore, inhibition of NF-κB nuclear translocation resulted in diminished PLK3 expression (Fig. 5C). An in silico analysis of human prostate tumor samples demonstrated a statistically significant inverse correlation of PLK3 and retinoblastoma (Fig. 5C) suggesting that retinoblastoma loss may prime towards a proapoptotic pathway. Overexpression of PLK3 in the setting of retinoblastoma deficiency inhibited growth (Fig. 5D, left and Supplementary Fig. S4A, left) and induced apoptosis (Fig. 5D, right and Supplementary Fig. S4A, right). Furthermore, knockdown of PLK3 in the retinoblastoma-deficient setting reversed this phenotype through decreased levels of apoptosis (Fig. 5E and Supplementary Fig. S4B). Thus, retinoblastoma loss results in activation of PLK3, a NF-κB–regulated gene that induces apoptosis and radiosensitivity.

**Retinoblastoma depletion results in marked in vivo radiosensitization**

To further mimic the clinical setting, the role of retinoblastoma in response to IR was explored in vivo. In retinoblastoma-proficient and retinoblastoma-deficient LNCaP cells were implanted into nude mice. As previously reported (10), no significant growth advantage was noted (Fig. 6A, left) between shCon and shRB LNCaP cells. After reaching 100 to 150 mm³, irradiation of the isogenic pairs unmasked a radiosensitivity advantage specific to retinoblastoma-deficient tumors (Fig. 6A, right). These data suggested that retinoblastoma depletion conferred a clear alteration in response to IR, as monitored by tumor growth kinetics; consensually, there was a significant decrease in tumor mass among shRB compared with shCon1 tumors at the time of sacrifice (Fig. 6B). In addition, serum PSA (also known as KLK3) was monitored. PSA is used clinically as a marker of prostate cancer detection, burden, and progression (30), and is not expressed in mice; thus, serum PSA was monitored as a measure of tumor growth. Serum PSA levels were significantly low in animals carrying the shRB xenografts (Fig. 6C). In addition, to determine the clinical impact of radiation sensitivity and retinoblastoma status, we retrospectively identified all cases of biopsy proven local recurrence following radiotherapy over the past 10 years. Eleven patients were identified (5 cases treated with external beam therapy and 6 treated with brachytherapy). All samples stained positively for retinoblastoma (Supplementary Fig. S5) further suggesting the diminished radiosensitivity of

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Figure 4. Retinoblastoma (RB) deficiency leads to activation of NF-κB pathway and cellular apoptosis in response to IR. A, immunoblotting analysis nuclear and cytoplasmic NFκB p50, NFκB p65, LaminB, GAPDH (left), and apoptotic marker cleaved caspase-3 (A, right), B, immunolocalization of nuclear NFκB p50 in retinoblastoma-proficient and retinoblastoma-deficient LNCaP cells 24 hours after radiotherapy (10 Gy). C, graphic representation of NFκB p50 binding to consensus sequence by TF ELISA in retinoblastoma-proficient and retinoblastoma-deficient LNCaP cells (10 Gy) in the presence of wild-type oligonucleotides and mutant oligonucleotides 24 hours after radiotherapy (10 Gy). D, growth curve and immunoblotting analysis of cytoplasmic and nuclear NFκB p50, NFκB p65, LaminB, GAPDH, and IκBα in LNCaP and LAPC4 shCon and shRB cells expressing IκBα DN. E, immunoblotting analysis of cleaved caspase-3 in LNCaP or LAPC4 shCon and shRB cells in the absence (left) and presence (right) of IR. F, immunolocalization of cleaved caspase-3 in LNCaP shRB cells with and without concurrent expression of IκBα DN in response to radiotherapy. Each data point is a mean ± SD from three or more independent experiments. *P < 0.05 were considered as statistically significant over shControl. Scale bar *-" = 20 μm.
Figure 5. Modulation of PLK3 alters cellular apoptosis through NF-κB-dependent manner. A, ChIP assay showing recruitment of NFκB p50 on the PLK3 promoter. B, microarray data validation of PLK3 and NOTCH2 (qRT-PCR; left) and immunoblotting analysis of PLK3 in shRB expressing IκBα-DN. C, in silico analysis of RB1 and PLK3 transcripts from human prostate tumor samples. Samples are ordered from low (blue) to high (red) RB1 and PLK3 expression (top). Boxplots show differential expression of PLK3 in normal prostate and tumor, grouped by retinoblastoma (RB) status (bottom; *: \( P = 1.46 \times 10^{-8} \) and statistically significant). D, cell growth and immunoblotting analysis of PLK3, cleaved caspase-3, and laminB in LNCaP shCon and shRB cells ectopically overexpressing PLK3. E, cell growth and immunoblotting analysis of PLK3, cleaved caspase-3, and laminB in PLK3-deficient LNCaP shRB cells. Each data point is a mean ± SD from three or more independent experiments. ***, \( P < 0.05 \) were considered as statistically significant over shControl.
tumors with intact retinoblastoma. The working model illustrates that NF-κB pathway drives the radiosensitized cells to cellular apoptosis through PLK3 (Fig. 6D). Collectively, these results indicate that active retinoblastoma promotes resistance to radiation, using both in vitro and in vivo models of disease progression.

Discussion

Given the frequency and importance of retinoblastoma inactivation in prostate cancer, understanding the response to radiotherapy is crucial for the development of effective therapeutic strategies and sequencing therapies. The present study identifies for the first time the impact of retinoblastoma status on radiation sensitivity. Key findings show that (i) regardless of hormonal environment, retinoblastoma loss sensitizes both hormone-sensitive and CRPC to IR both in vitro and in vivo; (ii) retinoblastoma loss alters multiple functionally important pathways critical in the response to radiotherapy including DNA damage and repair as well as apoptosis; (iii) modulation of NF-κB or PLK3 alters the cellular apoptosis and strongly suggests that retinoblastoma loss correlates with an increased nuclear translocation of NF-

Figure 6. Retinoblastoma (RB) deficiency results in marked in vivo radiosensitization. A, tumor volume analysis in LNCaP shCon and LNCaP shRB xenografts with no radiation (left) and after radiation (right). B, tumor mass analysis 1 to 5 weeks post-IR (5 Gy) in LNCaP shCon and LNCaP shRB xenografts. C, measurement of serum PSA levels 1 to 5 weeks post-IR. D, working model (schematics) shows the NF-κB-mediated apoptosis through PLK3 in retinoblastoma-deficient radiosensitized prostate cancer model. Each data point is a mean ± SD from 5 or more mice. **, P < 0.05 were considered as statistically significant.
kB resulting in cellular apoptosis mediated via PLK3 in response to radiotherapy.

The concept that retinoblastoma inactivation alters radiosensitivity via upregulation of apoptotic pathways as opposed to alterations in cell cycle was unexpected. DNA damage elicits arrest at both G1 and G2 phases of the cell cycle (31). G1 arrest occurs due to activation of the p53/p21 regulatory pathway and is dependent on functional retinoblastoma (32). Retinoblastoma is a known regulator of cell proliferation, and functional retinoblastoma is required to induce a cell-cycle arrest in G2 after DNA damage (9). G2 arrest generally relies on Chk1-dependent inactivation of the cyclin B1/Cdc2 kinase with maintenance of arrest after genotoxic stress being further regulated by p21, p53, and retinoblastoma (33). IR induces a G2 arrest in mouse embryo fibroblasts (MEF) with intact retinoblastoma, while MEF with knockout of retinoblastoma continue to proliferate and eventually undergo cell death (33). In the current study, there were no differential alterations in cell cycle noted between control and retinoblastoma knockdown prostate cancer cells after exposure to IR. However, retinoblastoma can inhibit cellular proliferation through distinct mechanisms: alterations in cell cycle and induction of cell death (34).

Retinoblastoma inhibits apoptosis in both normal tissue as well as tumor models. IR induces apoptosis in SAOS-2 cells, which lack functional retinoblastoma and this phenotype is reversed by stable transfection of retinoblastoma (35). Furthermore, E2F1, which is inactive when present in complex with retinoblastoma, is capable of inducing apoptosis (36). Prior studies have demonstrated that E2F1 and E2F3 are upregulated in the setting of retinoblastoma knockdown in prostate cancer (8, 36). Furthermore, etoposides sensitize retinoblastoma-deficient prostate cancer (8) and this is mediated via E2F1-mediated sensitivity to apoptotic stimuli. In this study, we demonstrated that proapoptotic pathways were upregulated after exposure to IR in the setting of retinoblastoma loss. Retinoblastoma inactivation primes cells for apoptosis via induction of procaspases (37). Caspase activation leads to apoptosis and increases the radiosensitivity of prostate cancer (38).

In the current study, cellular apoptosis was mediated via increased nuclear translocalization of NF-kB and induction of PLK3 and cleaved caspase-3. PLK3 is a NF-kB-regulated gene that induces apoptosis in both p53-dependent and independent signaling pathways (12). The function of NF-kB as either a proapoptotic or antiapoptotic signal is context dependent (39). In the context of LNCaP cells, increased levels of NF-kB activity led to increased apoptosis mediated via caspase activation (40). Docetaxel treatment also increases NF-kB activity in a dose-dependent manner leading to decreased cell survival in RWPE-2 prostate cells (41). Docetaxel and paclitaxel demonstrate enhanced sensitization to cell death in the context of retinoblastoma loss (10). Our study further clarifies that NF-kB-mediated apoptosis is regulated via PLK3 expression.

Notably, retinoblastoma loss is associated with altered AR activity and is causative for the transition to CRPC (8). Recent studies from our laboratory and others demonstrated that AR is a mediator of double-strand DNA break repair and can alter cell survival in response to DNA damage (42). Data herein demonstrate that retinoblastoma is dominant to these effects and that as mediated by deregulation of apoptotic signaling events, confers a robust radiosensitization phenotype.

Retinoblastoma status may be a viable biomarker from which to base therapeutic decisions (43). In tumors that retain retinoblastoma function, next-generation cyclin-dependent kinase (CDK) inhibitors may provide a robust approach to engage retinoblastoma tumor suppressor activity and halt cellular proliferation. Both preclinical (44) and early phase I trials demonstrate early tolerability and efficacy (44). For retinoblastoma-deficient cells, use of DNA-damaging agents is intriguing as this study confirms with previous reports that retinoblastoma loss confers hypersensitization to genotoxic stress (10, 45). Clinical observations from breast (46), bladder (47), and head and neck cancer (48) support the hypothesis that retinoblastoma-deficient cells have a compromised response to DNA damage. Within the context of clinical prostate cancer, retinoblastoma function has been incompletely defined due to the dual function of retinoblastoma loss increasing radiosensitivity while driving castrate-resistant growth (8). Low p16 expression, which is hypothesized to compromise retinoblastoma function, was associated with an increased risk of development of distant metastases in RTOG 9202, yet investigation of locally advanced disease indicates that loss of retinoblastoma and loss of p16 are not redundant (49, 50). Given that retinoblastoma loss-of-function is noted in the context of metastatic castrate-resistant prostate cancer (7), retinoblastoma may serve as a marker of response in the context of palliative radiation or radium-223 (11).

In summary, the findings herein present a paradigm for retinoblastoma function in protecting prostate cancer against IR. Retinoblastoma loss confers radiosensitivity via increased apoptosis. Given the resurgent role of radiation in the management of men with advanced castrate-resistant prostate cancer, a context in which retinoblastoma loss is common, retinoblastoma status may be a biomarker to therapeutic response.

Disclosure of Potential Conflicts of Interest
S.B. McMahon is a consultant/advisory board member for CellCentric Ltd. No potential conflicts of interest were disclosed by the other authors.

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