The retinoblastoma tumor suppressor modulates DNA repair and radioresponsiveness

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Running Title:
 RB loss alters therapeutic response

Key words: Retinoblastoma protein, ionizing radiation, DNA damage, apoptosis and prostate cancer

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Statement of conflict of interest: Authors have no potential conflicts of interest were disclosed.
Translational Relevance:

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

**Purpose:** Perturbations in the RB pathway are overrepresented in advanced prostate cancer; RB loss promotes bypass of first line hormone therapy. Conversely, preliminary studies suggested that RB-deficient tumors may become sensitized to a subset of DNA damaging agents. Here, the molecular and in vivo consequence of RB 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 RB 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 RB enhanced the radioresponsiveness of both hormone sensitive and castrate resistant prostate cancer. Hypersensitivity to ionizing radiation was not mediated by cell cycle or p53. RB loss led to alteration in DNA damage repair and activation of the NFκB pathway and subsequent cellular apoptosis through PLK3. *In vivo* xenografts of RB deficient tumors exhibited diminished tumor mass, lower PSA kinetics and decreased tumor growth after treatment with ionizing radiation (p<0.05).

**Conclusions:** Loss of RB 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 RB loss, but also credential RB status as a putative biomarker for predicting response to radiation therapy.
Introduction:

The retinoblastoma protein (RB1) is a tumor suppressor protein and is functionally inactivated in several major cancers (1). RB 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, RB is hypophosphorylated and forms a repressive transcriptional complex on E2F-regulated gene promoters to inhibit cell cycle. However, in response to mitogenic signals, RB phosphorylation disrupts the RB-E2F interactions facilitating G1/S cell cycle progression.

RB is a key regulator of multiple cellular functions including cell proliferation, apoptosis, differentiation, genome integrity, quiescence, senescence and DNA repair (3-6). RB 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 30-40% in metastatic or castration resistant prostate cancer (CRPC) samples (7). Loss of RB function, most commonly via allelic loss, facilitates development of resistance to hormone ablative therapies (8).

RB status has also been shown to alter response to genotoxic insults (9). Despite challenge with various chemotherapeutics, RB deficient mouse embryo fibroblasts failed to halt cell cycle progression resulting in incorrect DNA repair and cell death. However, within the context of prostate cancer cells, RB 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 RB depleted cells suggesting that cellular response to therapeutic intervention in prostate cancer cells is agent specific. Radiation therapy is a well-established treatment modality for localized and locally advanced prostate cancer. However, the role of radiation
therapy 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 RB inactivation, few studies have addressed the impact of this event on cellular response to ionizing radiation.

Herein, we delineated the impact of RB function on response to ionizing radiation using a panel of human isogenic prostate cancer lines with stable knockdown of RB. In this study, we show for the first time that loss of RB function results in increased radiosensitization of human prostate cancer cells, using both short-term growth as well as clonogenic survival assays. Further, the increased sensitivity is mediated through alterations in both apoptotic as well as DNA damage and repair pathways. Further the study identified a key mechanism of NFƙB 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 NFƙB 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 RB in regulating cell survival in prostate cancer after treatment with radiotherapy, and reveal the potential to personalize therapy prostate cancer patients based on RB status.
Materials and Methods:

Cell Culture. LNCaP and C4-2 cells were maintained in improved minimum essential medium (IMEM) supplemented with 5% FBS (heat-inactivated FBS). LAPC4 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% ΔFBS. 22Rv1 cells were maintained in RPMI supplemented with 10% FBS (Atlanta Biological, Flowery Branch, GA). 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, Logan, UT).

Immunofluorescence Analysis. Immunofluorescence staining was performed as previously described (10). Immunolocalization of γ-H2AX, 53BP1, cleaved caspase 3 and NFκBp50 was carried out by using a confocal microscopy (Nikon, Core Facility at Thomas Jefferson University).

Cell Growth Assay. RB proficient and deficient LNCaP, LAPC4, C4-2 and 22Rv1 cells were seeded at equal densities (1X10^5), exposed to ionizing radiation (PanTak Orthovoltage 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, Cambridge MA) or adenovirus harboring IκBα DN (SA mutation) (Vector Biolabs, Philadelphia, PA).

RNA Isolation and Microarray Analysis. Actively growing RB proficient and RB deficient LNCaP cells were exposed to ionizing radiation (10Gy) and the cells were harvested 24 hours post IR (three independent biological replicates). Total RNA was extracted using Trizol reagent (Invitrogen, Life Technologies, Grand Island, NY). 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 biological network and functional analyses and genes were highlighted. Microarray data was deposited in NCBI/GEO website and the GEO accession number GSE58711. p-values < 0.05 were considered as statistically significant. Microarray validation was conducted using qRT-PCR and quantified using a delta-delta CT method.

**Flow Cytometry Analysis.** Cell proliferation was assessed by BrdU incorporation using flow cytometry as previously described (14). Flow cytometry analysis was performed (GE Healthcare, Piscataway, NJ) and the data was 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, San Jose, CA), actin, LaminB, p21, NFκB p50, NFκB p65, IκBα cleaved caspase 3 (Santa Cruz Biotechnology Inc, Dallas, TX and Abcam, Cambridge, MA), PLK3 (Sigma-Aldrich, St. Louis, MO), DNA ligase IV (Abcam, Cambridge, MA), CDC25A, CDK2 and p53 (Santa Cruz Biotechnology Inc, Dallas, TX) by standard techniques and visualized using enhanced Western lightening chemiluminescence (Perkin-Elmer Life Sciences, Waltham, MA).

**Chromatin Immunoprecipitation Assay (ChIP).** Following ionizing radiation as described above, LNCaP and LAPC4 cells were cross-linked with formaldehyde and processed for
chromatin immunoprecipitation analysis as previously described (8). Equal concentrations of chromatin from all treatment groups were pre-cleared with protein Agarose beads in the presence of bovine serum albumin to reduce non-specific background. After removal of beads by centrifugation, 2 μg of NFκBp50 or NFκBp65 antibodies (Santa Cruz Biotechnology, Dallas, TX) were added and kept at 4°C for overnight on a rotary platform. The immunoprecipitated DNA was purified using PCR purification kit (Qiagen, Valencia, CA) and resuspended in 50 μl of sterile water. The purified DNAs and input DNAs were analyzed by semi quantitative PCR using PLK3 promoter targeting by using a forward 5’-GCC CGT GTC TAG CAT TTG AG-3’ and a reverse primers 5’-CCA TCA CAC CCG GCT AAT TT-3’ sequences, as described in (8, 12). Input DNA served as a positive control, where as rabbit IgG and a non-E2F/RB target albumin promoter were served as negative controls. PCR products resolved on agarose gel and the images were captured using BioRad HemiDoc Imager (Bio-Rad Laboratories Inc, Hercules, CA), the band intensities were measured using ImageJ and the results were presented graphically using GraphPad prism version 6 (GraphPad Software, Inc., La Jolla, CA). The qPCR results were quantitated using the delta-delta CT method.

**RB Knockdown:** RB knockdown was carried out as described in (8). Control and knockdown LNCaP, LAPC4, 22RV1 and C4-2 cells were generated through transfection with either shRNA plasmid directed against Rb (MSCV-Rb3C; targeted sequence: 5’-CGCATACTCCGGTTAGGACTGTATGAA-3’) or a control plasmid (MSCV donor) using Lipofectin transfection reagent (Invitrogen, Life Technologies, Grand Island, NY). Retrovirus encoding shRb plasmid (MSCV-LMP Rb88; targeted sequence: 5-GAAAGGCATGTGAACTTAA-3) or control plasmid (MSCV donor) were used to create RB-knockdown or control LAPC-4 stable clones. Following selection with puromycin for 6 to 7 days, stable clones were isolated and characterized. Puromycin selected cloned were subjected to RB mRNA (qRT-PCR) and
RB protein analysis (RB immunoblotting) and selected RB deficient clones were further utilized for the study.

**Comet Assay**: Alkaline comet assay was performed as described in (16) using the Trevigen comet assay kit (Trevigen Inc, Gaithersburg, MD). Slides were visualized using epifluorescence microscopy. The images were analyzed using CometScoring software (TriTek Corp, Sumerduck, VA).

**Transcription Factor Enzyme-Linked ImmunoSorbent Assay (TF-ELISA)**. TF ELISAs were performed as previously described (17). The protein-DNA complex was detected by chromogenic substrate. Absorbance of the samples was measured at 450nm using microplate reader (Bio-Tek I Spectrophotometer instruments, Winooski, VT).

**Xenografts and IR Treatments**: Xenografts were carried out as described previously (8). RB proficient and deficient LNCaP cells (4 × 10^6) were individually mixed (1:1) with matrigel in a 200 μ l volume (BD Biosciences, San Jose, CA) and the cells were implanted subcutaneously into the flanks of NCR/nu/nu (athymic) male mice. Once the tumors were reached 150 cubic mm volume and both RB proficient and RB deficient tumors were exposed to ionizing radiation (10 Gy, PanTakOrthovoltage X-ray irradiator). Tumor volumes were measured weekly with calipers, serum PSA levels were determined, and PSA doubling times were calculated. In shRB xenografts, maintenance of RB knockdown in vivo was verified by quantitative PCR (qRT-PCR) analysis of the human RB1 (shRB) transcript was performed as described in (8, 18). RB transcript levels as measured by qRT-PCR ranged from 10% to 90%. Maintenance of RB silencing was defined as having 30% or less of RB1 transcript level when compared to wild type xenograft tumors. Thirty percent of tumors failed to maintain loss of RB transcript and were not included in the analysis given the uncertainty of the timing when the tumors regained RB.
function. Animal studies were conducted in accordance with the principles and procedures outlined by the NIH guidelines and the IACUC of Thomas Jefferson University.

**PLK3 Ectopic Expression and knockdown**

PLK3 cDNA transfection and knockdown was carried as described in the manufactures protocol. Briefly 10 micrograms of PLK3 cDNA (Addgene, Cambridge MA) or control vector (PCDNA3) or PLK3 shRNA (Applied Biological Materials, Inc., Richmond, BC, Canada) 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, Grand Island, NY). The PLK3 over expression or PLK3 knockdown was confirmed by PLK3 immuno blotting. 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 caspase3 immunoblotting.

**Ectopic expression of dominant negative IκBαSA:** Adeno virus harboring dominant negative IκBαSA (Vector Biolabs, Philadelphia, PA) was infected in shRB cells (LNCaP and LAPC4) and exposed to ionizing radiation 10 Gy and processed for cell growth assay and cleaved caspase 3 apoptotic marker immunofluorescence and Western blotting analysis.

**RB IHC analyses.** RB IHC was performed as described in (8, 19). Eleven patients were identified in our institution who had biopsy proven local recurrence following primary radiation therapy (5 patients received external beam radiation therapy and received brachytherapy). Biopsy specimens or whole mount glands were used for IHC analyses. 5-μm sections were deparaffinized, antigen retrieval was performed in 10 mM EDTA (pH 9) for 10 minutes in a pressure cooker, and slides were incubated with 3% H₂O₂ for 10 minutes, then blocked with avidin/biotin blocking solution (Vector Biolabs, Philadelphia, PA) for 30 minutes and incubated in a 5% chicken/goat/horse serum solution for 2 hours. Sections were incubated with anti-RB
antibody overnight at 4°C [(4H1) Mouse mAb #9309, Cell Signaling Technology, Inc. Danvers, MA, USA; 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, Philadelphia, PA) for 30 minutes, developed using Vectastain ABC (Vector Biolabs, Philadelphia, PA) and stable DAB (Invitrogen, Life Technologies, Grand Island, NY) counterstained with hematoxylin, dehydrated, and mounted with Cytoseal XYL (Richard Allan Scientific, Thermo Fisher Scientific, Waltham, MA). The stain was interpreted as 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 (20X) and the scoring was performed by a clinical pathologist (an experienced staff pathologist) (Thomas Jefferson University Hospital, Philadelphia).

**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 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's correlation coefficient was calculated within normal prostate and prostate cancer groups. RB1 transcript levels were used to infer prostate cancer sample RB status, where RB-deficient samples were defined below the median RB1 expression, and RB-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 RB-proficient vs. normal and RB-deficient vs. RB-proficient groups.
**Statistics.** Statistical analyses were performed using GraphPad Prism (version 6.0) software (GraphPadPrismSoftware, Inc.). All the data were analyzed for statistical significance using Student’s t-test/ one-way ANOVA. For all experiments, p < 0.05 was considered statistically significant.
Results:

RB 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. RB inactivation occurs during prostate cancer progression and has been correlated with poor outcome (8). In human samples, RB loss of function occurs commonly via loss of heterozygosity (20). In order to delineate the role of RB in modulating the response to radiation therapy, 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). RB knockdown was verified using protein analysis across multiple prostate cancer cell lines (Figure 1A, B, left panel). First, in vitro clonogenic assays were performed. In the presence of androgen RB deficient LNCaP and LAPC4 cells showed increased radiosensitivity across all radiation doses studied, as compared to the isogenic RB proficient pairs. Statistical significance was observed after both 8 and 10 Gy exposure, indicating conservation of this result across multiple ranges of DNA damage (Figure 1C). Short-term growth assays using 10 Gy of ionizing radiation confirmed these findings, as control LNCaP and LAPC4 demonstrated greater viable cell number than their isogenic pairs (Figure 1A) in both hormone enriched and hormone free medium. Given that RB 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 (Figure 1B) in both hormone component and hormone free conditions. Together, these results suggest that RB loss sensitizes prostate cancer to ionizing radiation, 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.
The impact of RB on the radiation response is independent of p53. DNA damage induces cell-intrinsic checkpoints, including p21, p53, and RB (21). Further prior reports demonstrated that p53 and RB can exert partially overlapping roles in the setting of inactivation of either tumor suppressor (22). Thus, complementary up-regulation of p21 or p53 may occur in the setting of RB knockdown after exposure to ionizing radiation. In prostate cancer models, up-regulation and stabilization of p53 has been correlated with 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 (Supplemental Figure 1A and 1B) in either LNCaP or LAPC4 regardless of RB status. These findings indicate that cell proliferation and cell cycle control may be altered in RB knockdown cells given the lack of compensation for other prominent checkpoint regulators.

RB status does not impinge on the alterations of cell cycle after radiation. Given the prominent role of RB as a G1 cell cycle checkpoint through regulation of E2F family transcription factors, it was hypothesized that RB knockdown would alter cell cycle regulation after radiation. Further, in multiple cell lines with disrupted RB function, it has been consistently shown that RB deficiency allows cells to efficiently bypass the cell cycle inhibitory response to DNA damaging chemotherapeutic agents such as cisplatin (9). In both RB proficient and deficient prostate cancer cells, exposure to ionizing radiation resulted in a G1 cell cycle arrest. However, it was surprising that no differential response in cell cycle progression as measured by BrdU incorporation was noted after exposure to ionizing radiation regardless of RB status (Supplemental Figure 1C-F). No differences were observed in CDC25A expression regardless of RB status with or without exposure to ionizing radiation (Supplemental Figure 1G). Upregulation of CDK2 expression occurred in RB deficient cells, but was not altered by exposure to ionizing radiation (Supplemental Figure 1G). 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.

**RB status alters the transcriptional response to DNA damage.** Given that cell survival is a complex process and regulated by multiple underlying mechanisms, RB proficient and deficient LNCaP cells were exposed to ionizing radiation and followed by mRNA microarray analysis. After background correction and normalization, a gene list consisting of 1131 differentially (at least 1.5 fold change in expression) regulated genes were observed (Figure 2A). Using biological network and functional analysis, gene ontology demonstrated that 11 important functional pathways were significantly altered (Figure 2B) including cell death and survival, as well as DNA replication, recombination and repair, as a result of RB knockdown in the setting of DNA damage. An extended qRT-PCR validation was performed (Figure 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 RB deficient cells.

**RB loss alters DNA damage repair pathway.** Microarray transcript analysis demonstrated that DNA damage and repair pathway genes were elevated in RB deficient LNCaP cells (Figure 3A). Ionizing radiation results in multiple DNA aberrations including base excision as well as double standard DNA breaks(24) and microarray validation reveals that multiple base excision repair genes including POLB, POLK and REV3L (Figure 3B) as well as the DNA damage induced meiotic recombination gene SYCP3 (25) were up regulated and alternatively DNA ligase IV was down regulated (Figure 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 non-homologous end-joining pathway of double-strand break repair (26). These findings suggest that RB 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). γ-H2AX and 53BP1 foci were significantly elevated in shRB LNCaP cells as compared to control cells, indicating that radiation induced DNA damage depends on the RB status of the cells (Figure 3D left with quantification on right panel). The results were recapitulated in LAPC4 (Figure 3E panel), shRB cells showed a markedly diminished capacity to repair double strand breaks up to 24 hrs post-treatment. Comet assay recapitulated these findings demonstrating that RB deficiency radiosensitizes prostate cancer cells as shown with longer tail moments indicating higher amounts of DNA breaks (Figure 3F). Taken together, markers of DNA damage support the hypothesis that RB loss alters DNA repair mechanisms. Thus, this data reveals for the first time that RB promotes DNA double strand break repair independent of the ability of RB to regulate cell cycle progression.

**RB deficiency leads to activation of NFκB pathway and cellular apoptosis in response to ionizing radiation.** RB has been shown be function as an anti-apoptotic factor (28) and microarray analysis demonstrated that induction of pro-apoptotic transcripts in the setting of RB depletion (Supplemental Figure 2). Many of the altered gene transcripts are pro-apoptotic genes regulated via NFκB including DR4, TRAIL, RIP1 Fas, FasL, MYC, NOTCH2 and PLK3(29). The behavior of the transcription factor NFκB as a promoter or antagonist of apoptosis depends on the apoptotic stimulus. Higher total levels as well as increased nuclear translocation of NFκB p50 and p65 were observed in RB deficient cells in response to radiation (Figure 4A). Further immunolocalization and transcription factor ELISA analysis reveals that nuclear and DNA bound NFκB p50 was elevated in RB deficient LNCaP and LAPC4 cells (Figure 4B and C). In order to mimic NFκB knockdown, and we exogenously expressed a dominant negative IκBα (SA), which retains NFκB in the cytoplasm, thus preventing NFκB mediated downstream signaling. This modulation decreased nuclear translocation (Figure 4D, western blots) and decreased the radiosensitivity of shRB LNCaP and LAPC4 cells (Figure 4D,
growth curves). Transcription factor ELISA analysis reveals that nuclear and DNA bound NFκB p50 was diminished in RB deficient LNCaP and LAPC4 cells with the introduction of the dominant negative IκBα (Supplemental Figure 3A and B). Elevated levels of NFκB p50 and p65 protein have been demonstrated to activation of the apoptotic pathway (29). Knockdown of RB resulted in increased apoptosis in the setting of ionizing radiation (Figure 4E) and inhibition of NFκB via IκBα DN diminished cleaved caspase 3 levels (Figure 4F). Thus, RB loss results in upregulation and increased nuclear translocation of NFκB with subsequent induction of apoptosis in the setting of ionizing radiation.

**Modulation of PLK3 alters cellular apoptosis through an NFκB dependent manner.** NFκB 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. Chromatin immunoprecipitation assay and qRT-PCR revealed higher levels of NFκB p50 and p65 bound to the PLK3 promoter (Figure 5A). These increased binding correlated with increased expression of the PLK3 transcript in the setting of RB knockdown (Figure 5B). Further, inhibition of NFκB nuclear translocation resulted in diminished PLK3 expression (Figure 5C). An *in silico* analysis of human prostate tumor samples demonstrated a statistically significant inverse correlation of PLK3 and RB (Figure 5C) suggesting that RB loss may prime towards a pro-apoptotic pathway. Overexpression of PLK3 in the setting of RB deficiency inhibited growth (Figure 5D left panel, Supplemental Figure 4A left panel) and induced apoptosis (Figure 5D right, Supplemental Figure 4A right panel). Further, knockdown of PLK3 in the RB deficient setting reversed this phenotype through decreased levels of apoptosis Figure 5E, Supplemental Figure 4B). Thus RB loss results in activation of Plk3, a NFκB-regulated gene that induces apoptosis and radiosensitivity.
RB depletion results in marked in vivo radiosensitization. To further mimic the clinical setting, the role of RB in response to ionizing radiation was explored in vivo. RB proficient and RB deficient LNCaP cells were implanted into nude mice. As previously reported (10) and no significant growth advantage was noted (Figure 6A, left panel) between shCon and shRB LNCaP cells. After reaching 100–150 mm$^3$ irradiation of the isogenic pairs unmasked a radiosensitivity advantage specific to RB-deficient tumors (Figure 6A, right panel). These data suggested that RB depletion confers a clear alteration in response to ionizing radiation as monitored by tumor growth kinetics consonantly, there was a significant decrease in tumor mass among shRB compared with shCon1 tumors at the time of sacrifice (Figure 6B). Additionally, serum prostate-specific antigen (PSA; also known as KLK3) was monitored. PSA is used clinically as a marker of PCa 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 (Figure 6C). Additionally, in order to determine the clinical impact of radiation sensitivity and RB status, we retrospectively identified all cases of biopsy proven local recurrence following radiation therapy over the past 10 years. 11 patients were identified (5 cases treated with external beam therapy and 6 treated with brachytherapy). All samples stained positively for RB (Supplemental Figure 5) further suggesting the diminished radiosensitivity of tumors with intact RB. The working model illustrates that NFκB pathway drives the radio-sensitized cells to cellular apoptosis through PLK3 (Figure 6D). Collectively, these results indicate that active RB promotes resistance to radiation, using both in vitro and in vivo models of disease progression.
Discussion:
Given the frequency and importance RB inactivation in prostate cancer, understanding the response to radiation therapy is crucial for development of effective therapeutic strategies and sequencing therapies. The present study identifies for the first time the impact of RB status on radiation sensitivity. Key findings show that 1) Regardless of hormonal environment, RB loss sensitizes both hormone sensitive and CRPC to ionizing radiation both in vitro and in vivo; 2) RB loss alters multiple functionally important pathways critical in the response to radiation therapy including DNA damage and repair as well as apoptosis; 3) Modulation of NFκB or PLK3 alters the cellular apoptosis and strongly suggests that, RB loss correlates with an increased nuclear translocation of NFκB resulting in cellular apoptosis mediated via PLK3 in response to radiation therapy.

The concept that RB 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 RB (32). RB is a known regulator of cell proliferation and and functional RB is required to induce a cell cycle arrest in G1 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 RB (33). Ionizing radiation induces a G2 arrest in mouse embryo fibroblasts (MEF) with intact RB, while MEF with knockout of RB 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 RB knockdown prostate cancer cells after exposure to ionizing radiation. However, RB can inhibit cellular proliferation through distinct mechanisms: alterations in cell cycle and induction of cell death (34).
RB inhibits apoptosis in both normal tissue as well as tumor models. Ionizing radiation induces apoptosis in SAOS-2 cells, which lack functional RB and this phenotype is reversed by stable transfection of RB (35). Further, E2F1, which is inactive when present in complex with RB, is capable of inducing apoptosis (36). Prior studies have demonstrated that E2F1 and E2F3 are upregulated in the setting of RB knockdown in prostate cancer (8, 36). Further, etoposides sensitize RB deficient prostate cancer (8) and this is mediated via E2F1-mediated sensitivity to apoptotic stimuli. In this study, we demonstrated that pro-apoptotic pathways were upregulated after exposure to ionizing radiation in the setting of RB loss. RB 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 present study, cellular apoptosis was mediated via increased nuclear translocalization of NFκB and induction of PLK3 and cleaved caspase 3. PLK3 is a NFκB regulated gene that induces apoptosis in both p53 dependent and independent signaling pathways (12). The function of NFκB as either a pro-apoptotic or anti-apoptotic signal is context dependent (39). In the context of LNCaP cells, increased levels of NFκB activity led to increased apoptosis mediated via caspase activation (40). Docetaxel treatment also increases NFκB 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 RB loss (10). Our study further clarifies that NFκB mediated apoptosis is regulated via PLK3 expression.

Notably, RB 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 RB is dominant to these effects, and that as mediated by deregulation of apoptotic signaling events, confers a robust radiosensitization phenotype.

RB status may be a viable biomarker from which to base therapeutic decisions (43). In tumors that retain RB function, next generation cyclin-dependent kinases (CDK) inhibitors may provide a robust approach to engage RB tumor suppressor activity and halt cellular proliferation. Both preclinical (44) and early phase I trials demonstrate early tolerability and efficacy (44). For RB deficient cells, use of DNA damaging agents is intriguing as this study confers with previous reports that RB loss confers a hypersensitization to genotoxic stress (10, 45). Clinical observations from breast (46), bladder (47), and head and neck cancer (48) support the hypothesis that RB deficient cells have a compromised response to DNA damage. Within the context of clinical prostate cancer, RB function has been incompletely defined due to the dual function of RB loss increasing radiosensitivity while driving castrate resistant growth (8). Low p16 expression, which is hypothesized to compromise RB 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 RB and loss of p16 are not redundant (49, 50). Given that RB loss of function is noted in the context of metastatic castrate resistant prostate cancer (7), RB 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 RB function in protecting prostate cancer against ionizing radiation. RB loss confers radiosensitivity via increase apoptosis. Given the resurgent role of radiation in the management of men with advanced castrate resistant prostate cancer, a context in which RB loss is common, RB status may be a biomarker to therapeutic response.
Acknowledgements:
The authors thank members of the K. Knudsen laboratory for input and commentary. The authors specifically acknowledge M. Augello, J. Dean, M. Schiewer, R. DeLeuw for insightful discussion.

Grant Support:
The study was supported by a Young Investigator Award from the Prostate Cancer Foundation (to RBD), a Physician Research Training Award from the Department of Defense Grant (PC101841 to RBD), a PA Cure Award (to KEK), NIH grants (R01 CA176401 to KEK) and a Movember-PCF Challenge Award (to KEK).

Author’s Contributions:

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis: C. Thangavel, E. Boopathi, S. Ciment, R. Birbe, A. Ertel, S. Addya, P.M. Fortina.

Writing, review, and/or revision of the manuscript: C. Thangavel, K.E. Knudsen, R.B. Den, A.P. Dicker, S.B. McMahon.

References:

37. Williams D. Exclusive. Lamb to back integrated care 'experiments'. Health Serv J. 2012;122(6330):629-34.
Figure Legends:

Figure 1. 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 radiation therapy and processed for further analysis (A) RB Western blotting analysis in hormone responsive LNCaP and LAPC4 cells and actin loading control (top left panel). Cell number analysis of hormone responsive LNCaP and LAPC4 cells in hormone enriched and hormone free medium in response to radiotherapy (10 Gy) (top right panel). (B) RB Western Blotting analysis in castrate resistant C4-2 and 22Rv1 cells and actin loading control (bottom left panel). Cell number analysis of C4-2 and 22Rv1 cells in hormone enriched and hormone free medium in response to radiotherapy (10 Gy) (bottom right panel). (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. RB status alters the transcriptional response to DNA damage. Actively growing LNCaP and LAPC4 cells were exposed to radiation therapy and processed for further analysis (A) Microarray analysis (In silico) generated heat map of 1131 differentially regulated genes in RB proficient and deficient LNCaP cells 24 hours post IR (10 Gy). (B) IPA software generated deregulated functional pathways in RB proficient and deficient LNCaP cells in response to radiation therapy. (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. RB loss alters DNA damage and repair capacity in response to radiation. Actively growing LNCaP and LAPC4 cells were exposed to radiation therapy and processed for further analysis (A) In silico analysis generated heat map of DNA damage and repair pathway
genes from RB proficient and deficient LNCaP cells after 24 hours of post IR (10 Gy). (B) Microarray Validation of POLB, POLK, REV3L and SYCP3 transcripts in LNCaP cells exposed to IR. (C) Immunoblot analysis of DNA ligase IV in LNCaP and LAPC4 (shCon and shRB) cells in response to 10 Gy IR. (D) Confocal microscopic images of DNA damage induced γ-H2AX and 53BP1 foci (left panel) and a graphic representation of γ-H2AX and 53BP1 foci (right panel) in RB proficient and deficient LNCaP cells after post IR. (E) Confocal microscopic images of DNA damage induced γ-H2AX and 53BP1 foci (left panel) and a graphic representation of γ-H2AX and 53BP1 foci (right panel) in RB proficient and deficient LAPC4 cells after post IR (10Gy). (F) Photo micrographs of alkaline comet assay in LNCaP shCon, shRB LAPC4 shCon and shRB exposed to 20 Gy radiation (top panel) and graphic representation of alkaline comet assay (bottom panel). For each data point there is a mean ± SD from three or more independent experiments. **p <0.05 were considered as statistically significant. Scale bar “−” = 20 μm.

Figure 4. RB deficiency leads to activation of NFκB pathway and cellular apoptosis in response to ionizing radiation. (A) Immunoblotting analysis nuclear and cytoplasmic NFκB p50, NFκB p65, LaminB, GAPDH (left panel) and apoptotic marker cleaved caspase 3 (A, right panel) (B) Immunolocalization of nuclear NFκB p50 in RB proficient and RB deficient LNCaP cells after 24 hours of post radiation therapy (10 Gy). (C) Graphic representation of NFκB p50 binding to consensus sequence by TF ELISA in RB proficient and RB deficient LNCaP cells (10 Gy) in the presence of wild-type oligonucleotides and mutant oligonucleotides after 24 hours post radiation therapy (10Gy) (D) Growth curve and immunoblotting analysis of cytoplasmic and nuclear NFκBp50, NFκBp65, 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 panel) and presence (right panel) of ionizing radiation. (F) Immunolocalization of cleaved caspase3 in LNCaP shRB cells with and without
concomitant expression of \( \text{IkBa-DN} \) in response to radiation. 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 NFκB dependent manner.**

(A) Chromatin immunoprecipitation assay showing recruitment of NFκB p50 on the PLK3 promoter. (B) Microarray data validation of PLK3 and NOTCH2 (qRT-PCR) (left panel) and immunobloting analysis of PLK3 in shRB expressing IkBα-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 panel). Boxplots show differential expression of PLK3 in normal prostate and tumor, grouped by RB status (bottom panel, *\( p = 1.46 \times 10^{-5} \) and statistically significant). (D) Cell growth and immunoblot analysis of PLK3, cleaved caspase3 and laminB in LNCaP shCon and shRB cells ectopically overexpressing PLK3. (E) Cell growth and immunoblot analysis of PLK3, cleaved caspase3 and laminB in PLK3deficient 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.

**Figure 6. RB deficiency results in marked in vivo radiosensitization.** (A) Tumor volume analysis in LNCaP shCon and LNCaP shRB xenografts with no radiation (left panel) and after radiation (right panel). (B) Tumor mass analysis after 1-5 weeks of post IR (5 Gy) in LNCaP shCon and LNCaP shRB xenografts. (C) Measurement of serum PSA levels 1-5 weeks post IR. (D) Working model (schematics) shows the NFκB mediated apoptosis through PLK3 in RB deficient radio sensitized prostate cancer model. For each data point is a mean ± SD from 5 or more mice. **\( p < 0.05 \) were considered as statistically significant.
Figure 1.

(A) LNCaP

- LNCaP
  - shCon
  - shRB
  - RB
  - Actin
  - Lane 1, 2

(B) LAPC4

- LAPC4
  - shCon
  - shRB
  - RB
  - Actin
  - Lane 1, 2

(C) C4-2

- C4-2
  - shCon
  - shRB
  - RB
  - Actin
  - Lane 1, 2

- C4-2 (IR)
  - Relative Cell Number (Normalized to 0 Gy)
  - Hormone Proficient
  - Hormone Deficient

- Survival Fraction (LNCaP)
  - IR Dose (Gy)
  - Survival Fraction (LAPC4)
  - IR Dose (Gy)

- Survival Fraction (C4-2)
  - IR Dose (Gy)

- Survival Fraction (22Rv1)
  - IR Dose (Gy)
**Figure 2.**

### Table B: Biological Functions and P-values

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**Figure 2C:**

Expression of SOCS3, CYP3A5, BIK, TICAM1, IGFR1, and expression levels for shCon and shRB under IR (10 Gy) treatment.
Figure 3.

A) LNCaP + IR (10 Gy)

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B) Relative mRNA

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C) LNCaP and LAPC4

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DNA ligase IV

LaminB

IR 10 Gy

D) LNCaP

γ-H2AX 53BP-1 DAPI Merge

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Relative γ-H2AX Foci

Post-IR (hrs)

E) LAPC4

γ-H2AX 53BP-1 DAPI Merge

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Relative 53BP1 Foci

Post-IR (hrs)

F) Post-20 Gy IR (min)

LNCaP

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Median Tail DNA (%)

LAPC4

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Median Tail DNA (%)

Post-20 Gy IR (min)
Figure 4

A. LNCaP LAPC4

B. NFκB p50 DAPI Merge

C. OD 450 nm

D. LNCaP

E. IR 10 G

F. Cleaved CASP3 DAPI Merge
Figure 6.

A

B

C

D

Ionizing Radiation

NRB loss or inactivation

NFkB p50/p65

p50

p65

p50

p65

p50

p65

p50

p65

Nuclear Translocation

Hetero dimer formation

Transcription

Caspase 3 activation

Apoptosis

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The retinoblastoma tumor suppressor modulates DNA repair and radioresponsiveness

Chellappagounder Thangavel, Ettickan Boopathi, Steve Ciment, et al.

Clin Cancer Res Published OnlineFirst August 27, 2014.

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