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A Hormone–DNA Repair Circuit Governs the Response to Genotoxic Insult

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ABSTRACT

Alterations in DNA repair promote tumor development, but the impact on tumor progression is poorly understood. Here, discovery of a biochemical circuit linking hormone signaling to DNA repair and therapeutic resistance is reported. Findings show that androgen receptor (AR) activity is induced by DNA damage and promotes expression and activation of a gene expression program governing DNA repair. Subsequent investigation revealed that activated AR promotes resolution of double-strand breaks and resistance to DNA damage both in vitro and in vivo. Mechanistically, DNA-dependent protein kinase catalytic subunit (DNAPKcs) was identified as a key target of AR after damage, controlling AR-mediated DNA repair and cell survival after genotoxic insult. Finally, DNAPKcs was shown to potentiate AR function, consistent with a dual role in both DNA repair and transcriptional regulation. Combined, these studies identify the AR-DNAPKcs circuit as a major effector of DNA repair and therapeutic resistance and establish a new node for therapeutic intervention in advanced disease.

SIGNIFICANCE: The present study identifies for the first time a positive feedback circuit linking hormone action to the DNA damage response and shows the significant impact of this process on tumor progression and therapeutic response. These provocative findings provide the foundation for development of novel nodes of therapeutic intervention for advanced disease. Cancer Discov; 3(11):1254–71. ©2013 AACR.

INTRODUCTION

DNA damage response (DDR) pathways are intricate networks that sense DNA damage and activate repair mechanisms to maintain genomic integrity. Distinct DDR pathways exist to resolve different classes of DNA lesions, including mismatch repair, nucleotide excision repair, base excision repair, and the Fanconi anemia pathway (which resolves collapsed replication forks and associated interstrand crosslinks; ref. 1). Finally, DNA double-strand breaks, such as those induced by ionizing radiation, are repaired through one of two major pathways: nonhomologous end joining (NHEJ) or homologous recombination (HR; ref. 2). Predictably, dysregulation of such highly controlled repair processes can result in catastrophic cellular outcomes. Failure to efficiently repair DNA lesions reduces cellular viability via either apoptosis or senescence (3); alternatively, incomplete or inaccurate DNA repair promotes sustained protumorigenic DNA alterations (i.e., mutations, chromosomal losses/gains, and/or translocations; ref. 4).

Consonantly, DDR alterations are linked to tumor development and progression. Primarily, DDR and subsequent checkpoint activation serve as crucial mechanisms that maintain genomic stability and protect against gross genomic rearrangements that can lead to malignant conversion of normal cells (5, 6). However, deregulation of cell-cycle checkpoints in the presence of incomplete or inaccurate DDR results in inappropriate cell proliferation and survival; as such, mutations in DDR-associated genes are tightly associated with tumorigenesis (7). Failure to efficiently repair damage promotes errors in DNA replication and chromosome segregation, thus facilitating genomic instability in both hereditary and sporadic cancers (8). Direct ligation of double-strand DNA breaks through NHEJ also promotes deleterious rearrangements at the break site and chromosomal translocations associated with malignancy (9). Distinct from effects on tumor development, it is increasingly appreciated that heightened DDR can drive cancer progression and promote therapeutic resistance in established tumors, as DDR checkpoints are often disabled (5, 6). Notably, cancer cells that have acquired radioresistance show markedly enhanced DNA repair capacity (10). In addition, increased levels of key NHEJ components, including DNA-dependent protein kinase catalytic subunit (DNAPKcs), Ku70, and Ku80, correlate with recurrence after radiotherapy and progression to advanced disease (11, 12). Recent findings across multiple tumor types have shown that DNA repair genes are upregulated in primary tumors predicted to metastasize, indicating that heightened DNA repair capacity is associated with increased risk of distant metastasis (13). On balance, emerging evidence strongly supports the contention that escalated DDR function contributes to disease progression and therapeutic resistance. As such, concentrated effort has been put forth to determine how these alterations may lead to therapeutic opportunities and to delineate the molecular mechanisms controlling DNA damage repair in human malignancies.

Unexpectedly, clinical observations suggest that steroid hormones might modulate the response to DNA damage in hormone-responsive cancers. In breast cancer, chemotherapeutics...

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that induce genotoxic stress are used to treat all known subtypes, including those that remain dependent on estrogen receptor (ER) alpha (14). Clinically, suppression of ER signaling by tamoxifen enhances the response to radiation (15); although the underlying mechanisms have not been completely defined, ER can interact with a subset of known DDR factors (16) and modulate expression of several genes associated with DDR (17). Prostatic adenocarcinoma, another example of a hormone-responsive cancer, is classified according to disease stage and is dependent on androgen receptor (AR) activity at all stages for growth, survival, and progression (18). Upon binding of ligand (androgens), AR is activated, releases from inhibitory heat-shock proteins, homodimerizes, and translocates to the nucleus, where it binds to androgen response elements (ARE) on DNA and induces a gene expression program that is requisite for prostatic adenocarcinoma maintenance (19). AR-directed therapeutics play a central role for all stages of prostatic adenocarcinoma. In the context of disseminated prostatic adenocarcinoma, although the vast majority of patients respond initially to androgen deprivation therapy (ADT), the response is unfortunately transient, and recurrent, castration-resistant prostatic adenocarcinoma (CRPC) tumors arise within 24 to 36 months due to inappropriate AR reactivation (20). Importantly, although AR frequently is activated in CRPC under castrate conditions, the receptor still responds to androgen, including those produced intratumorally (21). Treatment of CRPC also includes DNA-damaging agents such as radium-223 chloride, which targets to bone metastases and emits alpha radiation; however, as monotherapy, this agent affords only a modest survival advantage (22), clearly defining the need for improved and synergistic approaches to effectively treat advanced disease.

Among men diagnosed with localized prostatic adenocarcinoma, one of the primary treatment modalities is radiotherapy; however, similar to metastatic disease, it is clear that those patients with high-risk or locally advanced disease have suboptimal outcomes when treated with radiation alone (23). In the context of locally aggressive disease, the combination of ADT and radiation is the standard of care, based on numerous phase III randomized trials encompassing thousands of patients, which show large survival benefits with this combination therapy compared with either treatment alone (24–26). Although these trials indicate that AR activity may impact tumor sensitivity to radiation, the mechanism underlying this cooperativity has not been defined. Molecularly, AR activation has been associated with the creation of DNA double-strand breaks at sites of transcriptional regulation, incurred as a way to release torsional strain and facilitate gene expression (27). Moreover, DNAPKcs and Ku70/80 have been preliminarily proposed using in vitro models to serve as AR coactivators (28). Thus, despite clinical evidence to suggest cross-talk between AR and DDR pathways, the mechanism and consequence of this communication is not understood.

Given these observations and the urgent need to identify durable mechanisms to manage advanced disease, the present study aimed to investigate the influence of activated AR on the DDR and resultant biologic outcomes. Findings herein reveal that activated AR promotes expression and activity of key factors involved in DDR. Critical observations show that radiotherapy induces AR activity, and that AR promotes resistance to DNA damage, using both ADT-sensitive and CRPC models of disease. Elucidation of the underlying mechanism revealed that AR activity promotes DNA double-strand break resolution, independent of effects on cell cycling, by regulating expression and activity of key players in DDR. AR is recruited to regulatory regions of the genes identified, directly implicating AR as a transcriptional regulator of components of the DDR. Further mechanistic investigation identified AR-activated DNAPKcs expression and function as a critical component of AR-mediated DNA repair and cellular survival after genomic insult. Moreover, accumulated DNAPKcs feeds back to AR and supports the transcriptional transactivation potential of the receptor. The collective findings suggest a model wherein androgen-induced DNAPKcs expression and activity enhance AR activity, creating a positive circuit through which androgens promote DNA repair and tumor cell resistance to DNA damage-inducing therapeutics. As will be shown, these studies identify activated AR as a key regulator of the DDR, and characterize a novel mechanism of regulation that reveals new opportunities for precision therapies in AR-dependent tumors.

RESULTS

Suppression of AR Activity Enhances the Response to DNA Damage

To explore the impact of androgens on DDR, prostatic adenocarcinoma cells were exposed to ionizing radiation in combination with androgen-replete (control) or androgen-deprived conditions mimicking ADT. In ADT-sensitive, AR-positive models of human disease (LNCaP and LAPC4), control cells exhibited expected growth kinetics in the presence of androgen, but exposure to ionizing radiation treatment alone reduced cell survival and doubling by approximately 50%, consistent with the known role of ionizing radiation in suppressing prostatic adenocarcinoma growth (Fig. 1A, top). In parallel, the ADT-sensitive cancer models showed a strong antiproliferative response to ADT alone, exemplified by a 2.3-fold increase in cell-doubling time (Fig. 1A, bottom). Despite the robust response to ADT alone, combining ADT and ionizing radiation further modestly suppressed cell growth and survival at both 6 and 10 days after treatment compared with untreated control, ADT, or ionizing radiation alone (Fig. 1A, top). Consonantly, combination treatment further increased cell doubling time, showing a potential cooperative effect between ADT and ionizing radiation (Fig. 1A, bottom). Alteration of treatment schedule did not significantly alter cell number (Supplementary Fig. S1A and S1C), similar to what was observed clinically (29). In contrast, combination therapy resulted in no significant alteration in cell number (as compared with ionizing radiation alone) in AR-negative prostatic adenocarcinoma cells (PC3; Fig. 1A, top and bottom). Combined, these studies suggest that androgen deprivation in ADT-sensitive cells and resultant suppression of AR activity modestly enhances the response to radiation in vitro. To interrogate this concept in vivo, xenografts of ADT-sensitive disease were randomized into one of four treatment arms (control, ionizing radiation, castrate, or castrate and ionizing radiation), treated, and tumor volume monitored (Fig. 1B, top). Although all three treatment arms resulted in delayed
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Androgen ablation cooperates with ionizing radiation (IR) in hormone therapy (HT)-sensitive prostatic adenocarcinoma (PCa). A, top, cells were cultured in hormone-proficient (FBS, full serum control) media for 24 hours and then treated with 2 Gy IR, steroid-deprived conditions (ADT), or a concurrent combination of ADT and 2 Gy IR. Cell number was determined on days 6 and 10 posttreatment and set relative to day 1 (control, untreated). Bottom, relative cell-doubling time was calculated on day 10 for each treatment using the formula \( T_d = \frac{(t_2 - t_1) \times \ln(q_2/q_1)}{\ln(2)} \), where \( t \) is time and \( q \) is quantity. B, top, schematic representing xenograft treatment cohorts. Bottom, LNCaP cells were injected into the flanks of nude mice and randomized into one of the four treatment arms as shown. Tumor volume was determined periodically and relative volume reported.* \( P < 0.05 \) compared with all other treatment conditions.

Figure 1. Androgen ablation cooperates with ionizing radiation (IR) in hormone therapy (HT)-sensitive prostatic adenocarcinoma (PCa). A, top, cells were cultured in hormone-proficient (FBS, full serum control) media for 24 hours and then treated with 2 Gy IR, steroid-deprived conditions (ADT), or a concurrent combination of ADT and 2 Gy IR. Cell number was determined on days 6 and 10 posttreatment and set relative to day 1 (control, untreated). Bottom, relative cell-doubling time was calculated on day 10 for each treatment using the formula \( T_d = \frac{(t_2 - t_1) \times \ln(q_2/q_1)}{\ln(2)} \), where \( t \) is time and \( q \) is quantity. B, top, schematic representing xenograft treatment cohorts. Bottom, LNCaP cells were injected into the flanks of nude mice and randomized into one of the four treatment arms as shown. Tumor volume was determined periodically and relative volume reported.* \( P < 0.05 \) compared with all other treatment conditions.

tumor growth compared with control, the combination of ADT and ionizing radiation resulted in more effective tumor suppression compared with either modality alone (Fig. 1B, bottom). Together, these data show that androgen ablation can cooperate with ionizing radiation to modestly decrease tumor cell growth and survival in ADT-sensitive, AR-positive prostatic adenocarcinoma.

AR Suppression Sensitizes CRPC to DNA Damage

Although the observation that suppression of AR activity can enhance the response to radiation shows in vitro and in vivo mimicry of clinical outcomes, it is well established that ADT induces a potent G1 arrest in hormone therapy-sensitive cells (30), which could therefore impact efficacy of the radiation-induced DNA damage response. Consequently, the subsequent studies were conducted using ADT-refractory prostate cancer cells (castrate-resistant prostate cancer cells), which fail to undergo G1 arrest upon androgen ablation and/or AR suppression, and represent advanced disease. Initial in vitro growth analyses were conducted in CRPC models that attained ADT resistance through variant mechanisms. Notably, although C4-2 cells express full-length AR, 22Rv1 cells express full-length and short forms of AR (derived from alternative splicing) that lack the ligand (androgen) binding
ADT alone had little influence on CRPC cells, whereas ionizing radiation resulted in an approximately 50% reduction in overall cell survival relative to control (Fig. 2A, left). Strikingly, combining ADT and ionizing radiation resulted in dramatically enhanced growth suppression (Fig. 2A, left) and increased cell-doubling time by more than threefold in both model systems as compared with ADT alone (Fig. 2A, right).

Alteration in treatment schedule resulted in only minimal changes in cell number (Supplementary Fig. S1B and S1C). These findings indicate that the impact of androgens and AR on the response to radiation occurs independently of AR-dependent cell-cycle control, and is highly significant in models of late-stage, castration-resistant disease.

To further assess the role of AR activity on the response to DNA damage, dihydrotestosterone (DHT) resupplementation...
studies were carried out. As shown in Fig. 2B, cells subjected
to steroid deprivation and then supplemented with DHT
during radiation showed extensive rescue of cell growth and
proliferation, as shown by a nearly twofold reduction in doubling
time compared with combined ADT and ionizing radiation,
and only a slight increase compared with ionizing radiation
alone (Fig. 2B). These provocative findings strongly suggest
that androgens and AR activity promote resistance to geno-
toxic insult. To further assess this posist, additional studies
were conducted using the next-generation AR antagonist
MDV3100 (enzalutamide), which not only competitively
blocks DHT for binding to the receptor, but also promotes cyto-
plasmic sequestration of AR (32). In these studies, CRPC cells
treated with MDV3100 responded similarly to ADT alone,
exhibiting only a marginal decrease in cell number compared
with control (Fig. 2C). Consistent with results obtained from
ADT treatment, combining MDV3100 and ionizing radiation
resulted in substantially decreased cell number and subse-
quent increase in cell-doubling time compared with control
or either treatment alone (Fig. 2C). Furthermore, xenographs
of CRPC models were randomized into one of four treatment
arms (control, ionizing radiation, castrate, or castrate and
ionizing radiation) as depicted in Fig. 2D, treated, and tumor
volume monitored. Consistent with in vitro findings, castra-
tion alone had minimal effect on tumor volume, whereas the
combination of castration and ionizing radiation resulted in
profound growth suppression (Fig. 2D, bottom). Collectively,
these results indicate that active AR promotes resistance to
radiation, using both in vitro and in vivo models of disease
progression.

DNA Damage Induces AR Activity

To begin to explore the mechanism by which AR promotes
radiation resistance, AR activity was assessed in CRPC cells
after ionizing radiation treatment by analyzing expression
of clinically relevant AR target genes (KLK3/PSA, TMPRSS2,
and FKBP5) whose expression is known to be under strin-
tent AR regulation. As shown in Fig. 3A, both TMPRSS2 and
FKBP5 transcripts showed marked induction after radiation,
whereas KLK3/PSA levels were generally unchanged (Fig. 3A).
Furthermore, treatment with increasing dosage of ionizing
radiation resulted in elevated expression of AR target genes
(Supplementary Fig. S2), further suggesting that AR activ-
ity is selectively enhanced in response to genomic insult.
To address the specificity of this response, the impact of
doxorubicin (known to result in double-strand DNA breaks),
was assessed. As shown, marked induction of AR activity
occurred, similar to that observed in response to ionizing
radiation (Fig. 3B). Interestingly, treatment with UV, which
primarily results in single-strand DNA breaks, failed to alter
AR target gene expression (Fig. 3B). Finally, as DNA damage
is known to induce reactive oxygen species (ROS), the impact
of a ROS scavenger, N-acetylcysteine, was assayed. Pretreat-
ment of cells with N-acetylcysteine before ionizing radiation
reversed the observed induction of AR target genes, thus
implicating ROS and free radicals generated by DNA damage
as being involved in the response (Fig. 3C). Combined, these
observations unexpectedly showed that AR activity is selec-
tively induced in response to DNA double-strand breaks in
a dose-dependent manner, and that this event involves ROS
generation. The specificity of this event was further assessed
in parallel studies, and it was observed that androgen depriva-
tion nullified the DNA damage–induced upregulation of AR
target gene expression (Fig. 3D, left). Consistent with effects
on cell growth and survival, DHT resupplementation res-
cued gene expression after ionizing radiation (Fig. 3D, left).
In contrast, cells cultured in hormone-privileged conditions
and treated with MDV3100 showed gene expression profiles
similar to those cultured under conditions of androgen depriva-
tion, with no detectable increase in AR target gene expres-
sion observed after ionizing radiation treatment (Fig. 3D,
right). DHT supplementation was unable to influence AR
target gene expression in cells treated with MDV3100, likely
because the low level of DHT added (0.1 nmol/L) was unable
to outcompete MDV3100 (1 μmol/L) for binding to AR (32).
Combined, these results show that AR activity is induced by
DNA damage in models of CRPC.

AR Promotes DNA Double-Strand
Break Resolution

It is well established that cell-cycle checkpoints are induced
in response to radiation that allow for evaluation of the extent
of damage, and subsequent DNA repair or cell death ensues
(33). Given the robust observations that AR activity promotes
cell survival after DNA damage, it was critical to assess the
impact of AR on radiation-induced cell-cycle alteration and
cell death. First, to assess checkpoint control, BrdUrd incor-
poration indices and cell-cycle profiles were assessed after
radiation exposure, in both the presence and the absence
of androgen, and after androgen deprivation with DHT resup-
plementation. Reduction in BrdUrd incorporation and cell-
cycle arrest were observed as an initial response to radiation
in all three conditions (Fig. 4A, left, and Supplementary Fig. S3A
and S3B). Similarly, reentry into the cell cycle, as observed
by monitoring BrdUrd kinetics after radiation, was relatively
equivalent amongst the three conditions. Next, to assess the
impact on cell death, sub-G1 cell populations were quantified
for each condition after radiation (Fig. 4A, right). No signifi-
cant differences in sub-G1 cells were observed, suggesting that
AR activation status does not significantly influence rates of
cell death in CRPC cells. This is distinct from what has been
previously observed in a model of hormone therapy–sensitive
disease (34), revealing distinctions in CRPC after radiation.
Thus, in advanced disease the function of AR in promoting
cell survival after DNA damage seems to occur independently
of alterations in cell-cycle control or cell death. As such, the
impact of AR on DNA repair was determined.

A proximal consequence of radiation is DNA damage,
the most lethal of which is DNA double-strand breaks (35).
Inability to repair DNA double-strand breaks is known to be
detrimental to cell growth and survival (3, 36). To determine
the impact of AR on this process, the proficiency of CRPC
cells to repair DNA double-strand breaks in the presence or
absence of androgen, or after steroid deprivation and DHT
resupplementation, was assessed using γ-H2AX and 53BP1
foci as markers of double-strand breaks (37). As expected,
stereoid deprivation alone did not alter γ-H2AX and 53BP1
foci formation, as compared with untreated controls (Fig. 4B
and C). In contrast, radiation exposure resulted in rapid
induction of γ-H2AX (∼11.5 foci/cell at 2 hours after ionizing
**Figure 3.** DNA damage induces AR activity. **A,** C4-2 cells were cultured in hormone-proficient media for 24 hours then treated with 2 Gy ionizing radiation (IR). **B** and **C,** C4-2 cells were cultured in hormone-proficient media for 24 hours then treated with 2 Gy IR, 100 J/m² UV, 1 μmol/L doxorubicin, or 1 hour pretreatment of 1 μmol/L N-acetylcysteine (NAC) followed by 2 Gy IR. **D,** left, C4-2 cells were cultured in hormone-deficient or hormone-deficient supplemented with 0.1 nmol/L dihydrotestosterone (DHT) media for 24 hours then treated as indicated. Right, C4-2 cells were cultured in hormone-proficient media for 24 hours then treated with 1 μmol/L MDV3100, concurrent 1 μmol/L MDV3100 and 2 Gy IR, or concurrent 1 μmol/L MDV3100, 2 Gy IR, and 0.1 nmol/L DHT. Relative expression of indicated transcript levels were analyzed and normalized to GAPDH mRNA then set relative to untreated control at indicated time points after treatment. *, P < 0.05; **, P < 0.01. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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**Figure 4.** AR promotes DNA double-strand break resolution. **A**, C4-2 cells were cultured in hormone-proficient media for 24 hours then treated as indicated. At indicated time points, cells were pretreated with BrdUrd for 2 hours and then fixed. Cells were incubated with BrdUrd antibody and propidium iodide and processed for fluorescence-activated cell sorting analysis. Data is graphed as normalized to untreated control. **B** and **C**, C4-2 cells were cultured in hormone-proficient media for 24 hours then treated as indicated. Cells were fixed at the indicated time points, stained with α-γ-H2AX and α-53BP1 antibodies, and imaged by confocal microscopy. Foci were counted and plotted as average foci per cell set relative to control at each time point. **D**, C4-2 cells were cultured in hormone-proficient, hormone-deficient, or hormone-deficient supplemented with 0.1 nmol/L DHT media for 24 hours then treated with 15 Gy ionizing radiation and harvested at indicated time points. Single-cell gel electrophoresis was conducted and tail moments assessed.

*P < 0.05;** P < 0.01.
radiation, consistent with levels previously reported for similar doses and time courses; ref. 37) and 53BP1 foci (indicative of DNA double-strand breaks) compared with control (Fig. 4B and C). Cells treated with ionizing radiation alone (in hormone-proficient conditions) showed double-strand break resolution at 22 hours posttreatment (Fig. 4B and C), consistent with what has previously been reported for similar doses of ionizing radiation (38). Remarkably, cells deprived of hormones just before radiation and held in androgen-free conditions after radiation showed a markedly diminished capacity to repair double-strand breaks 22 hours posttreatment. In fact, elevated levels of both γH2AX and 53BP1 foci persisted up to 72 hours (Fig. 4B and C), indicating that cells subjected to androgen deprivation were severely compromised in the ability to repair radiation-induced double-strand breaks. The specificity of this event was further shown in that cells subjected to steroid deprivation but resupplemented with physiologically relevant levels of DHT showed restoration of double-strand break repair at 22 hours after treatment (Fig. 4B and C). To further interrogate the hypothesis that activated AR promotes DNA break repair, analysis of DNA fragmentation after high-dose ionizing radiation (Fig. 4B and C). To further interrogate the hypothesis that activated AR promotes DNA break repair, analysis of DNA fragmentation by neutral Comet assay revealed significantly elevated DNA fragmentation after high-dose ionizing radiation (15 Gy) in cells deprived of hormone, an effect that was reversed by resupplementation with DHT (Fig. 4D). Combined, these data confirm that AR activation results in repair of DNA damage. These unexpected findings reveal for the first time that AR promotes DNA double-strand break repair, independent of the capacity of AR to regulate cell-cycle progression and cell survival.

**AR Activity Regulates Genes Required for DNA Damage Repair**

Given the well-defined role of AR as a transcription factor (39) combined with the new observations herein that: (i) DNA damage induces AR activity; (ii) active AR promotes cell survival after DNA damage; and (iii) AR facilitates double-strand DNA break repair, the impact of AR on expression of genes encoding the DNA repair machinery was determined. A rigorous screen was developed wherein a custom quantitative PCR (qPCR) array was used to assess changes in the DNA damage and repair-associated transcriptome after radiation, in the presence and absence of hormone (Fig. 5A, left). The screen was implemented to identify genes significantly up- or downregulated by both radiation and androgens (see Supplementary Fig. S4A), and genes with identical patterns in both of the CRPC models tested were prioritized for further analyses. Using this strategy, three lead candidates were identified: PRKDC (encoding the protein product DNAPKcs), XRCC2, and XRCC3 (Fig. 5A, right). These results were notable, as DNAPKcs binds broken DNA ends through the Ku70/80 heterodimer and is critical for proper recruitment of repair factors during NHEJ (1, 40), whereas XRCC2 and XRCC3 are RAD51 paralogs that promote strand transfer at sites of DNA damage during HR (41). As each was induced by androgen in the presence of DNA damage, previously published chromatin immunoprecipitation (ChIP)-seq data (39), in addition to our own ChIP-seq analyses (McNair and colleagues, manuscript in preparation), were used to identify AR binding within 50 Kb of the transcriptional start site for each gene, as would be expected on the basis of current knowledge of AR function (42). Using these combined datasets, AR-occupied regions (AROR) were identified in the putative regulatory regions of all three genes, and validation studies were initiated using this information. For each potential AROR, ChIP analyses were conducted at multiple time points posttreatment in either hormone-proficient or hormone-deficient conditions. For XRCC2, two ARORs were identified, both of which contained an ARE half site (43). As shown, AR was recruited in a time-dependent manner (post–ionizing radiation) both in hormone-proficient conditions and in cells which had been deprived of steroids and resupplemented with DHT (Fig. 5B, red and orange lines). Recruitment to these sites was diminished in hormone-deficient conditions after ionizing radiation (Fig. 5B, purple line), indicating that increasing AR activity promotes binding after ionizing radiation. For XRCC3, only one AROR was identified (containing an ARE half site), and although the recruitment of AR was not as striking as that exhibited by XRCC2, elevated AR recruitment at 16 hours post–ionizing radiation was observed only in hormone-proficient conditions (Fig. 5C, red line). Finally, similar to XRCC2, two ARORs were identified within proximity of the PRKDC locus; however, neither contained an ARE half site. This is consistent with previous studies, which reveal that AREs are present at only approximately 40% of known AR-binding sites (44). Again, AR recruitment to these sites was observed in a time-dependent fashion after ionizing radiation treatment in both hormone-proficient and hormone-deficient supplemented with DHT conditions (Fig. 5D, red and orange lines). AR recruitment was reduced in hormone-deficient conditions after ionizing radiation (Fig. 5D, purple line), suggesting activated AR acts as a critical regulator of gene expression in response to ionizing radiation. No substantial recruitment of AR relative to control was detected at a region of the KLK3/PSA locus known to be devoid of AR-binding sites (ref. 45; Supplementary Fig. S4B), suggesting that elevated levels of AR recruitment to the DNA damage loci represent specific recruitment of AR in response to DNA damage. Collectively, the DNA damage gene expression profile combined with the AR chromatin binding data support the novel concept that AR activity regulates genes required for DNA damage repair.

**AR Induces DNAPKcs Expression and Activity**

Of the genes identified, PRKDC is the most critical for efficient DNA repair, exhibiting a well-defined role in the early stages of the NHEJ double-strand break repair pathway (1, 40). As cells were unable to efficiently repair double-strand breaks when AR activity was suppressed, the impact of AR status on DNAPKcs expression was investigated. As shown, compared with ADT or combination ADT and ionizing radiation, supplementation with DHT in either treatment, respectively, resulted in induction of overall DNAPKcs levels (Fig. 6A; compare lanes 1, 2, and 5, 6, quantification on right). Treatment with MDV3100 also modestly diminished DNAPKcs levels compared with combination MDV3100 and DHT treatment (Fig. 6A; compare lanes 3, 4, quantification on right). Furthermore, CRPC cells treated exclusively with DHT showed increased expression of DNAPKcs compared with untreated control (Fig. 6B; compare lanes 1, 2, quantification on right). Cells treated with ionizing radiation showed
**Figure 5.** AR activity regulates genes required for DNA damage repair. A, left, schematic showing experimental design. Right, array results depicting genes regulated by androgen after 2 Gy ionizing radiation (IR) in C4-2 and 22Rv1 cells. OR, occupied region. B–D, C4-2 cells were cultured in hormone-proficient, hormone-deficient, or hormone-deficient supplemented with 0.1 nmol/L DHT media for 24 hours then treated as indicated. Samples were harvested for ChIP-qPCR analysis and percent (input) occupancy of AR set relative to control at each time point is reported. Arrows and numbers on diagrams depict location of identified AROR in relation to transcriptional start site (TSS).

Regulated by androgen after IR.
Figure 6. AR induces DNAPKcs expression and activity. A and B, C4-2 cells were cultured in hormone-proficient, hormone-deficient, or hormone-deficient supplemented with 0.1 nmol/L DHT media for 24 hours then treated. Cells were harvested and expression of DNAPKcs was analyzed and quantified. Representative image of at least three independent experiments is shown. C and D, same as A and B, but expression of phospho (Ser2056)-DNAPKcs was analyzed and quantified. DNAPKcs-specific activity was determined by dividing phospho-DNAPKcs by total DNAPKcs. E, C4-2 cells were transfected with a pool of siRNAs directed against the XRCC6 transcript or control siRNA for 96 hours then treated with 2 Gy ionizing radiation (IR). Cells were harvested and expression of DNAPKcs, phospho-DNAPKcs, and Ku70 was analyzed and quantified. *, P < 0.05.
A similar trend regarding DNAPKcs levels compared with cells treated with ionizing radiation and DHT despite not achieving statistical significance (Fig. 6B; compare lanes 3, 4, quantification on right). These data indicate that activated AR promotes DNAPKcs expression in both the presence and absence of ionizing radiation.

In addition to examining DNAPKcs expression, phosphorylation of DNAPKcs on Ser2056 (indicative of activated DNAPKcs) was interrogated in all treatment conditions, as DNAPKcs activity is known to be induced in response to DNA damage (46). CRPC cells treated with ADT or MDV3100 showed decreased phospho-DNAPKcs levels and decreased DNAPKcs-specific activity (phospho-DNAPKcs/total DNAPKcs) compared with control, which was rescued by supplementation with DHT (Fig. 6C; compare lanes 1, 2 and 2, 3). Finally and most intriguingly, the combination of ADT and ionizing radiation elicited decreased phospho-DNAPKcs and DNAPKcs-specific activity compared with ionizing radiation alone, a result that was reversed by supplementation with DHT (Fig. 6D; compare lanes 1, 2 and 2, 3). To further address the mechanism(s) by which AR controls DNAPKcs activity, Ku70 was depleted by siRNA, as Ku70 has been shown to recruit DNAPKcs and contribute to DNAPKcs activation (47). Moreover, the gene encoding Ku70 (XRCC6) was identified as a potential AR target gene in the qPCR array (Supplementary Fig. S4A). As shown, Ku70 depletion resulted in decreased phospho-DNAPKcs in response to ionizing radiation treatment (Fig. 6E), revealing one mechanism by which AR likely controls DNAPKcs activation. Overall, these data reveal that an activated AR signaling axis induces both DNAPKcs expression (through binding to and regulating the PRKDC locus) and activity (requiring Ku70), in both the presence and the absence of DNA damage.

DNAPKcs Is Required for DNA Repair in the Presence of Androgen

To determine whether altered DNAPKcs expression is sufficient to account for the inefficient double-strand break repair and decreased cell number observed after combination ADT and ionizing radiation in CRPC cells, DNAPKcs was depleted by RNA interference and double-strand break resolution assessed by quantification of γ-H2AX and 53BP1 foci formation (Fig. 7A). DNAPKcs knockdown alone did not induce DNA double-strand breaks (Fig. 7A; compare green line with blue line). Consistent with Fig. 4, control cells treated with ionizing radiation exhibited increased levels of both γ-H2AX and 53BP1 foci immediately following ionizing radiation treatment, and the lesions were largely resolved within 24 hours (Fig. 7A, red line). However, cells with diminished DNAPKcs expression exhibited both an increase in foci immediately following ionizing radiation and a significantly compromised ability to repair double-strand breaks, noted by elevated levels of foci that persisted for up to 72 hours post–ionizing radiation (Fig. 7A; compare purple and red lines). These results are consistent with the concept that AR-mediated double-strand DNA break repair is dependent on AR-induced DNAPKcs expression and activity. DNAPKcs is also required for AR-mediated cell survival after DNA damage, as combined DNAPKcs knockdown and ionizing radiation decreased cell number compared with siControl, siControl and ionizing radiation, or DNAPKcs knockdown alone (Fig. 7B). Importantly, DNAPKcs knockdown persisted throughout the course of the experiment (Supplementary Fig. S5), and these results mimic growth suppression seen in CRPC cells after combination ADT and ionizing radiation treatment (Fig. 2A), indicating that loss of DNAPKcs expression at least partially accounts for the observed decrease in cell number. In addition, DNAPKcs knockdown caused significant decreases in expression of AR target genes (Fig. 7C), consistent with previous reports identifying DNAPKcs as a transcriptional coactivator of AR (28). Consistent with what has previously been reported (48), DNAPKcs depletion resulted in decreased expression of ATM and a concurrent decrease in phospho-ATM levels (Supplementary Fig. S6A).

If AR-driven DNAPKcs expression and activity are critical for AR-mediated repair and survival post–ionizing radiation, heightened AR activity may be able to overcome DNAPKcs inhibition. To further address this hypothesis, a highly specific DNAPKcs inhibitor (Nu7441; ref. 49) was assessed for the impact on AR-dependent DNA repair and cellular outcomes. First, cells cultured in hormone-proficient conditions were exposed to DHT for 1 hour followed by treatment with the DNAPKcs inhibitor and ionizing radiation. At 24 hours after treatment, cells treated with the DNAPKcs inhibitor and ionizing radiation exhibited elevated levels of γ-H2AX and 53BP1 foci compared with cells treated with ionizing radiation alone (Fig. 7D). Remarkably, cells pretreated with DHT showed a significant reduction in foci levels (though rescue of repair capacity was only partial), indicating that an active AR signaling axis resulted in enhanced DNA double-strand break repair (Fig. 7D). Accordingly, cells pretreated with DHT exhibited modestly increased cell number 5 days after treatment compared with cells that were treated with DNAPKcs inhibitor and ionizing radiation alone (Fig. 7E). DNAPKcs inhibition also resulted in increased levels of phospho-ATM without significantly altering overall ATM levels (Supplementary Fig. S6B), consistent with what has been reported regarding redundancy between DNAPKcs and ATM in activating the damage response (50). To further assess the requirement of DNAPKcs as a modulator of AR-mediated outcomes, clonogenic assays were conducted (Supplementary Fig. S7A–S7E). Similar to in vitro cell-doubling assays in Fig. 1, the impact of androgen deprivation on the response to ionizing radiation was modest in the hormone therapy–sensitive models (Fig. S7A), consistent with previous reports (34), and no effect of variant hormone status was observed in AR-negative cells (Fig. S7B). In contrast, a robust effect was observed in CRPC cells, which was rescued by DHT supplementation (Fig. S7C and S7D). Strikingly, suppression of DNAPKcs activity eliminated the radioprotective impact of androgens in these models, although pretreatment with DHT again partially restored the protective effect (Fig. S7E). Additional analysis showed that pretreatment with DHT resulted in enhanced repair within 2 hours of post–ionizing radiation exposure (Supplementary Fig. S8A and S8B), showing an immediate impact on repair kinetics. Analysis of HR activity, monitored by Rad51 foci, revealed a significant increase in HR activity at 2, 5, and 8 hours after ionizing radiation treatment (Supplementary Fig. S8C), showing that AR activation results in a rapid increase in HR-mediated repair when DNAPKcs is inhibited. Furthermore, use of a plasmid-based NHEJ activity assay showed that activation
**Figure 7.** DNAPKcs is required for DNA repair in the presence of androgen. **A**, C4-2 cells were transfected with a pool of siRNAs directed against the PRKDC transcript or control siRNA for 96 hours then treated with 2 Gy ionizing radiation (IR). Cells were fixed at the indicated time points, stained with α-γ-H2AX and α-53BP1 antibodies, and analyzed by confocal microscopy. Foci were counted and plotted as average foci per cell set relative to control at each time point. **B**, same as A, but cells were counted 5 days after treatment and cell number set relative to control. **C**, same as A, but relative expression of transcript levels was analyzed and normalized to GAPDH mRNA at 24 hours after treatment. **D**, C4-2 cells were cultured in hormone-proficient media for 24 hours then treated with combinations of DHT (1 hour pretreatment), 2 Gy IR, and 1 μmol/L DNAPKcs inhibitor. Cells were fixed at the indicated time points, stained with α-γ-H2AX and α-53BP1 antibodies, and analyzed by confocal microscopy. Foci were counted and plotted as average foci per cell set relative to control at each time point. **E**, same as D, but cells were counted 5 days posttreatment and cell number set relative to control. **F**, C4-2 cells transfected with linearized pEYFP plasmid were treated as indicated and activity assessed. **G**, model describing the feedback circuit of AR and DNAPKcs expression and activity controlling DNA repair and tumor cell survival *+, P < 0.05; **+, P < 0.01 value. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DSB, double-strand break.
of AR resulted in a significant increase in NHEJ activity, but only a modest increase in NHEJ when DNAPKcs is inhibited (Fig. 7F). In sum, these results identify DNAPKcs as an important effector of AR function whose activity is critical for efficient AR-mediated DNA repair. Combined, the data presented suggest an overall model wherein AR signaling directly regulates DNAPKcs expression and indirectly influences DNAPKcs activity, resulting in a positive feedback circuit controlling efficient DNA double-strand break repair after DNA damage and tumor cell survival (Fig. 7G).

**DISCUSSION**

Understanding the mechanisms by which altered DDR promotes tumor progression and aggressive phenotypes is crucial for development of effective therapeutic strategies for advanced cancers. The present study identifies for the first time a critical link between hormone action and DNA repair. Key findings show that (i) DNA damage enhances AR activity; (ii) androgens promote double-strand DNA break repair and resistance to genotoxic insult both in vitro and in vivo; (iii) DNAPKcs expression and activity are induced by androgens; (iv) AR-mediated DNA repair requires DNAPKcs activity; and (v) DNAPKcs enhances AR activity, thereby creating a positive feedback circuit through which androgens promote DNA repair. Together, these findings uncover a powerful and unexpected mechanism through which AR promotes tumor cell survival and therapeutic resistance, and identify a new node for therapeutic intervention.

The concept that DNA damage activates AR and promotes AR binding to genes whose products promote DNA repair was unexpected. With few exceptions, there is little understanding of the cause and consequence of DNA damage-induced transcription factor activation. The p53 tumor suppressor is phosphorylated and activated by multiple DDR factors in response to DNA damage (51) and alters the cellular response to genotoxic insult. Although the effects of AR activity on the response to DNA damage were consistent in both p53-positive (e.g., LNCaP) and p53-deficient models (e.g., LAPC4), p53 has been proposed to influence AR activity (52), and may therefore contribute to altered AR activity after genotoxic insult. The E2F1 transcription factor is also induced in response to DNA damage (53) and can promote double-strand DNA break repair (53); strikingly, E2F1 positively regulates AR and is frequently upregulated in CRPC (54), providing an additional means by which AR activity may be induced in response to DNA damage. Alternatively, the histone acetyltransferases CBP and p300, known AR coactivators, promote expression of the HR genes BRCA1 and RAD51 (55), indicating that AR likely uses a distinct cohort of cofactors to alter gene expression after DNA damage. Finally, posttranslational modifications of nucleosomes are known to influence DDR (56), and it is also tempting to speculate that AR may be recruited to genes that promote DDR via altered histone modification. Combined, these collective studies strongly suggest that DNA damage-induced transcription factor activation plays a major role in DDR and the cellular response to genotoxic damage.

DNA damage-activated AR was found herein to promote expression of numerous genes associated with DNA repair (Fig. 5 and Supplementary Fig. S2). Flap endonuclease 1 (FEN1), which is implicated in base excision repair and oxidative stress response (57), is associated with aggressive prostatic adenocarcinoma (58). As ionizing radiation induces oxidative damage in addition to DNA double-strand breaks, increased FEN1 expression after DNA damage may contribute to radiotherapy resistance via modulation of the oxidative stress response, a concept supported by the finding that ROS and free radicals generated by damage play a role in the response of AR to damage (Fig. 3C). More proximal to DNA double-strand break repair, XRCC6 (protein product Ku70) is induced by AR after DNA damage. Ku70 binds the free ends of DNA double-strand breaks as a heterodimer with Ku80 and recruits DNAPKcs to facilitate NHEJ (1, 40). Recent reports show that Ku70 expression is decreased after castration and correlates with PSA in clinical specimens (59), providing additional evidence that AR regulates Ku70 in human disease. In addition to FEN1 and XRCC6, genes whose products are central to DNA double-strand break repair were identified herein as regulated by AR after DNA damage. XRCC2 and XRCC3, each RAD51 paralogs required for RAD51 recruitment and accumulation during HR, are both induced in the presence of androgens after DNA damage. Additional analysis revealed AR recruitment to regulatory regions of both genes in a time-dependent fashion post–ionizing radiation only when androgens were present. Although AR-regulated expression of XRCC2 has not been previously reported, XRCC3 was recently identified as an AR target gene in CRPC tissue (60). Furthermore, XRCC3 was identified as part of a 16-gene signature that predicts CRPC (60), indicating that AR-mediated expression of XRCC3 (and possibly XRCC2, as both genes have similar functions) drives resistance to therapy and development of late-stage disease. Time-dependent recruitment of AR to XRCC2 and XRCC3 regulatory regions observed herein in response to ionizing radiation reveals a novel signaling network where AR induces XRCC2 and XRCC3 as a direct response to DNA damage. Although ongoing investigation will delineate the contribution of these AR-regulated genes to AR-mediated DNA repair and therapeutic resistance, among the AR targets identified herein, PRKDC (protein product DNAPKcs) plays the most prominent role in the DDR.

DNAPKcs initiates NHEJ after recruitment to DNA double-strand breaks, and the present study clearly identifies AR-mediated DNAPKcs expression and activity as essential for AR-mediated DNA repair and therapeutic resistance. Strikingly, little is understood with regard to DNAPKcs regulation—basal levels of genes involved in NHEJ (including PRKDC) are expressed throughout all stages of the cell cycle (61). In breast cancer, ER was recently shown to alter DNAPKcs expression, in that activated ER binds sites within regulatory regions of PRKDC, however, whether this event occurs after DNA damage remains uncertain (17). In contrast, the data presented herein show for the first time that activated AR resulted in increased DNAPKcs expression, and that AR was recruited to regulatory regions of PRKDC in a time-dependent manner after DNA damage. These findings reveal the crucial mechanistic insight that AR is a transcriptional regulator of DNAPKcs expression. Moreover, the critical role of DNAPKcs in DNA double-strand break repair identifies AR-driven DNAPKcs expression as an important mediator of therapeutic resistance. Although AR directly binds and promotes expression of the PRKDC locus,
active AR also stimulates DNAPKcs activity, as determined by increased phospho-DNAPKcs and DNAPKcs-specific activity. Furthermore, inhibition of DNAPKcs activity resulted in the inability to repair DNA damage and decreased cell growth. However, both effects were partially reversed by increased AR-mediated induction of HR, as shown by increased Rad51 foci and, to a minor extent, NHEJ activity. The ability of AR to induce HR is not entirely surprising, given the finding that AR induces XRCC2 and XRCC3, two genes involved in HR-mediated repair, in response to ionizing radiation (Fig. 5B and C). In addition, as HR is known to require a template for repair, AR-mediated cell-cycle progression stimulated by DHT provides an environment that is required for HR, ultimately further implicating AR in the coordination of DDR pathways. Combined, these findings reveal that though AR-mediated DNA repair is complex, DNAPKcs activity is essential for efficient AR-mediated repair of damaged DNA and resultant cell survival. DNAPKcs is typically autophosphorylated in response to ionizing radiation and recruited to broken DNA ends by the Ku70/Ku80 heterodimer, where additional phosphorylation events occur (62). Interaction between Ku70 and DNAPKcs suggests a possible mechanism of AR-mediated DNAPKcs regulation; in addition to recruiting DNAPKcs to broken DNA ends, Ku70 can facilitate DNAPKcs phosphorylation (47), and this is supported by the finding that Ku70 depletion resulted in decreased phospho-DNAPKcs in response to ionizing radiation treatment in CRPC cells (Fig. 6E).

Furthermore, the observations that DNAPKcs inhibition and DNAPKcs knockdown both resulted in inefficient DNA double-strand break repair despite exerting opposite effects on phospho-ATM levels strongly suggest that AR-mediated DNA repair is dependent on DNAPKcs but not directly on ATM. On balance, the data herein intriguingly show that AR controls both DNAPKcs expression and activity, and that efficient AR-mediated DNA repair requires DNAPKcs activity.

Although the main function of DNAPKcs is as a mediator of DDR, it is becoming well appreciated that DNAPKcs impinges on other cellular processes (63). Specifically, DNAPKcs can function as a transcriptional coactivator for numerous hormone receptors, including ER and PR (progesterone receptor; refs. 16, 64). DNAPKcs directly phosphorylates ER, facilitating protein stability and transcriptional activation (16). Although little is known with regard to AR, DNAPKcs can induce AR transcriptional activity in a luciferase reporter system (28), suggesting that DNAPKcs may serve as an AR coactivator. Consistently, DNAPKcs downregulation resulted in a significant decrease in expression of numerous AR target genes, indicating that DNAPKcs is necessary for robust AR activity. Combined, these data show that AR-driven DNAPKcs expression and activity is required for AR-mediated DNA repair and that DNAPKcs enhances AR transcriptional activation, resulting in a positive feedback circuit that drives tumor survival and therapeutic resistance.

Given the role of DDR in cancer progression, and recent revelations that advanced-stage prostate cancers show significant chromosomal alterations (65), considerable efforts have been put forth to develop therapeutic strategies targeting double-strand break repair, focusing on precision approaches based on pathways driving different tumor types (66). The discovery of a positive feedback circuit involving AR-mediated expression and activity of DNAPKcs and DNAPKcs activation of AR reveals that targeting AR in combination with DNAPKcs inhibition and a DNA damaging agent may be an effective therapeutic strategy in CRPC as well as other AR-driven cancers including liver (67) and molecular apocrine breast cancer (68); this postulate is currently under investigation. Targeting the AR-DDR crosstalk is dependent on the ability to decrease AR activity and disrupt DDR by targeting factors critical for damage repair, though diminishing AR activity in CRPC has proven challenging. However, next-generation AR antagonists such as enzalutamide and ARN-509 have shown significantly enhanced efficacy in late-stage disease (32, 69). In addition, intratumoral androgen synthesis is targeted using the CYP17A inhibitor abiraterone acetate, resulting in decreased AR activity in CRPC (70). Related to the present findings, recently developed DNAPKcs inhibitors are now in phase I trials for multiple, advanced, solid tumors (66). In the context of AR-dependent cancers, it is hypothesized that DNAPKcs inhibitors would both diminish DDR capacity and reduce the transcriptional activation of AR-dependent cell survival. Thus, targeting the dual roles of DNAPKcs is likely to suppress multiple pathways that promote advanced tumor phenotypes.

In summary, the present study identifies a positive feedback circuit linking hormone action to the DNA damage response, and shows the significant impact of this process on tumor progression and therapeutic response. These provocative findings provide the foundation for development of novel nodes of therapeutic intervention for advanced disease.

METHODS

Cell Culture and Treatment

See Supplementary Materials and Methods. Cell lines used were not cultured longer than 6 months after receipt from the original source of American Type Culture Collection.

Cell Growth Assays

Assays were conducted as described previously (71). Media was changed on day 6 for all 10-day cell growth studies.

Xenograft Analysis

Cells were resuspended in 100 μl of saline with 50% Matrigel (BD Biosciences) and injected subcutaneously into the flank of 6-week-old athymic nude male mice (NCI-Frederick, Frederick, MD). Treatment was initiated when tumors reached approximately 150 mm³, as described in Supplementary Materials and Methods. Tumor volume was measured periodically and at time of sacrifice with digital calipers. Mice were sacrificed once their tumors reached an approximate size of 1,000 mm³. Mouse weight was assessed at time of sacrifice and no mice lost more than 5% of their initial body weight.

Gene Expression Analysis

Cells were seeded at equal densities in hormone-proficient or hormone-deficient conditions. RNA was isolated using TRIzol and cDNA generated using SuperScript III (Invitrogen). Quantitative PCR was conducted using an ABI StepOne machine and PowerSybr in accordance with the manufacturer’s specifications using primers described in Supplementary Table S1.

Flow Cytometry

Cells were seeded in hormone-proficient media and labeled with BrdUrd (1:1,000) 2 hours before harvest. Cells were fixed in ice-cold 100% ethanol and stained with fluorescein isothiocyanate-conjugated
anti-BrdUrd antibody (BD Biosciences, #5560288). Ethanol-fixed cell pellets were gently resuspended in 0.1 mL PBS containing 0.02 mg/mL propidium iodide and 0.04 mg/mL RNase A, incubated for 15 minutes at room temperature in the dark, and processed using a Beckman FACs Calibur (at least 10,000 events per sample). Analysis was conducted using FlowJo (v8.8) for BrdUrd incorporation and cell-cycle profile.

**Immunofluorescence Analysis**

Cells were seeded onto coverslips and fixed in 3.7% formaldehyde at indicated time points. Cells were permeabilized in 0.3% TritonX-100 at 37°C for 30 minutes then stained with primary antibody (1:500) for 1 hour at 37°C, followed by secondary antibody (1:1,000) for 1 hour at room temperature, using antibodies described in Supplementary Materials and Methods. All immunofluorescence was counterstained with DAPI to visualize nuclei, and coverslips were mounted on slides using Gelvatol. Foci were imaged on a Zeiss Confocal Laser Scanning microscope, counted for at least 50 cells per treatment condition, and set relative to untreated control.

**Comet Assay**

Single-cell gel electrophoresis was conducted according to the manufacturer's instructions (Trevigen). See Supplementary Materials and Methods for experimental details.

**PCR Array Analysis**

Cells were seeded in hormone-proficient or hormone-deficient conditions and treated as indicated. RNA was isolated using TRIzol and cdNA generated using the RT² First Strand Kit (SABiosciences) according to manufacturer's specifications. cdNA was analyzed by real-time PCR using either the DNA Repair (#PAHS-042Z) or DNA Damage Signaling Pathway (#PAHS-029Z) RT² Profiler PCR array (SABiosciences) and results quantified according to manufacturer's specifications.

**ChIP Analysis**

Cells were seeded in hormone-proficient or hormone-deficient conditions and treated as specified. Cells were fixed with 1% formaldehyde at indicated time points and ChIP analyses conducted as previously described (54). Genomic DNA was purified and quantitative PCR conducted for indicated loci using primers described in Supplementary Table S1. Data were analyzed as percentage of input total samples calculated as previously described (54).

**Immunoblotting**

Cells were seeded in hormone-proficient or hormone-deficient conditions and treated as specified. Cell lysates (30–40 μg) were generated as previously described (72), resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed using antibodies described in Supplementary Materials and Methods. Quantification was conducted using a Bio-Rad ChemiDoc MP Imaging System.

**RNA Interference**

Cells were seeded at a density of 1 x 10^4 in hormone-proficient conditions (complete media) for 24 hours. Cells were then transfected (6–8 hours) in serum-free conditions with either control, PRKDC, or XRCC6 siRNA pools (Thermo Scientific, D-001810-10-20, L-005030-00-0005, respectively) according to the manufacturer’s specifications. Cells were maintained in complete media for an additional 96 hours then treated as specified and harvested at indicated time points. For growth analysis, a day 1 count was taken 96 hours after transfection for both siPRKDC and siControl cells to which day 5 cell counts were compared. Immunoblot analysis to confirm DNA-PKcs or Ku70 knockdown was conducted on cells harvested at time of treatment (96 hours after transfection).

**NHEJ Activity Assay**

Linearized pEYFP vector was transfected into C4-2 cell lines. The cells were treated with either DHT (0.1 or 1 nmol/L) or DNAPKcs inhibitor (NU7441 1 μmol/L) at the time of transfection. Twenty-four hours after transfection, genomic DNA was extracted with Puregene Cell and Tissue kit (Cat. No.158388). Quantitative PCR was conducted using an ABI StepOne machine and PowerSybr in accordance with the manufacturer’s specifications using primers described in Supplementary Table S1. The relative efficiency of NHEJ was calculated by the comparative Ct method according to the Ct, values of the internal control as described previously (73).

**Clonogenic Survival Assays**

See Supplementary Materials and Methods.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.F. Goodwin, M.J. Schiewer, J.L. Dean, R.S. Schregoest, R. de Leeuw, T. Ma

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.F. Goodwin, M.J. Schiewer, J.L. Dean, R.S. Schregoest, R. de Leeuw, R.B. Den, A.P. Dicker, F.Y. Feng, K.E. Knudsen

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A Hormone–DNA Repair Circuit Governs the Response to Genotoxic Insult


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