The convergence of DNA damage checkpoint pathways and androgen receptor signaling in prostate cancer

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Abstract

It is increasingly clear that castration-resistant prostate cancer (PCa) is dependent on the androgen receptor (AR). This has led to the use of anti-androgen therapies that reduce endogenous steroid hormone production as well as the use of AR antagonists. However, the AR does not act in isolation and integrates with a milieu of cell-signaling proteins to affect cell biology. It is well established that cancer is a genetic disease resulting from the accumulation of mutations and chromosomal translocations that enables cancer cells to survive, proliferate, and disseminate. To maintain genomic integrity, there exists conserved checkpoint signaling pathways to facilitate cell cycle delay, DNA repair, and/or apoptosis in response to DNA damage. The AR interacts with, affects, and is affected by these DNA damage-response proteins. This review will focus on the connections between checkpoint signaling and the AR in PCa. We will describe what is known about how components of checkpoint signaling regulate AR activity and what questions still face the field.

Introduction

Prostate cancer (PCa) is the second most common cancer among men worldwide, with over 1.1 million new cases of PCa reported in 2012 (www.cancer.org). When PCa initially presents in the clinic, the tumor is dependent on androgen for growth; therefore, for patients presenting with disseminated PCa, the tumor is responsive to therapies that take advantage of depletion of circulating androgens. However, therapeutic success is temporary with the tumor invariably recurring as a metastatic and castration-resistant disease.

A unique characteristic of PCa is the continual dependence on the androgen receptor (AR) for growth and survival (Jenster 1999, Culig & Bartsch 2006). The AR is a member of the nuclear receptor superfamily that acts as a transcription factor (Evans 1988, Mangelsdorf *et al.* 1995,

Gelmann 2002). Upon androgen binding, there is a change in the complement of AR-associated proteins as the AR shuttles to the nucleus and binds as homodimers to androgen-response elements (AREs) within the regulatory regions of target genes. The recruitment of co-activators and co-repressors and chromatin remodeling promote the induction of AR-dependent gene transcription (Heinlein & Chang 2002). The specific combination of cofactors that are recruited to AREs provides for tissue- and ligand-specific gene expression. Given the fundamental role of AR action in prostate development and homeostasis, one can envision why AR function is coopted in PCa to promote tumor cell growth and survival (Feldman & Feldman 2001, Balk 2002).

The acquisition of genome instability through the accumulation of mutations and chromosomal translocations

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Endocrine-Related Cancer

is an emerging hallmark of cancer that enables cancer cells to survive, proliferate, and disseminate (Hanahan & Weinberg 2011). Physical and chemical environmental agents, such as u.v. light, ionizing radiation (IR), and chemotherapy, and normal cellular processes, including DNA replication, all assault cellular DNA and induce up to 10^5 lesions daily (Hoeijmakers 2009). To maintain genomic integrity, phyllogenetically conserved check-point signaling pathways are activated to facilitate cell cycle delay, DNA repair, and/or apoptosis (Zhou & Elledge 2000, Bartek et al. 2007, Ciccia & Elledge 2010). These checkpoint networks must detect the damage and rapidly transmit the signal to downstream effector proteins.

This review focuses on the connections between checkpoint signaling and the AR in PCa. We explore what is known about how components of DNA damage signaling regulate AR activity, what questions still face the field, and how answering these questions may shape how patients are treated.

DNA damage checkpoint signaling in PCa

The DNA damage response (DDR) is composed of a sophisticated signal transduction sub-network that encompasses transcriptional regulation, checkpoints, apoptosis, and DNA repair mechanisms, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and classical nonhomologous end-joining (cNHEJ) (Bartek et al. 2007). Recently, another DNA repair mechanism that has been discovered in cNHEJ-proficient cells goes by several names: alternative NHEJ (aNHEJ), microhomology-mediated end joining (MMEJ), and backup NHEJ (bNHEJ) (Deriano & Roth 2013). Occurring mainly in cells deficient for components of cNHEJ, aNHEJ frequently results in excessive deletions and chromosomal translocations (Zhu et al. 2002, Guirouilh-Barbat et al. 2007, Yan et al. 2007, Boboila et al. 2010, Simsek & Jasin 2010). While the factors involved and mechanism underlying aNHEJ are poorly understood, both poly (ADP-ribose) polymerase 1 (PARP1) and the MRN (MRE11, RAD50, NBS1) complex appear to play key roles. Recent work has linked the MRN complex to the process of end resection and PARP1 to join at telomeres lacking the Ku heterodimer (Haince et al. 2008, Deriano et al. 2009, Rass et al. 2009, Xie et al. 2009, Sfeir & de Lange 2012). Further studies need to be performed to determine whether aNHEJ operates as an independent repair pathway. We refer the reader to a recent review for more details on aNHEJ (Boboila et al. 2012).

Different endogenous (reactive oxygen species, stalled replication forks) and environmental insults (u.v., IR, chemotherapy) cause various types of DNA damage (single-strand breaks (SSBs), double-strand breaks (DSBs), chemical adducts, mismatches). In response to cellular insults, the activation of the DDR is initiated by the phosphorylation of the family of phosphatidylinositol-3-kinase-related kinases ataxia telangiectasia mutated (ATM), ATM and RAD-related (ATR), and DNA-dependent protein kinase (DNA-PK), which is composed of the regulatory KU70/80 heterodimer and catalytic subunit DNA-PKcs (Fig. 1). These kinases, in turn, activate the
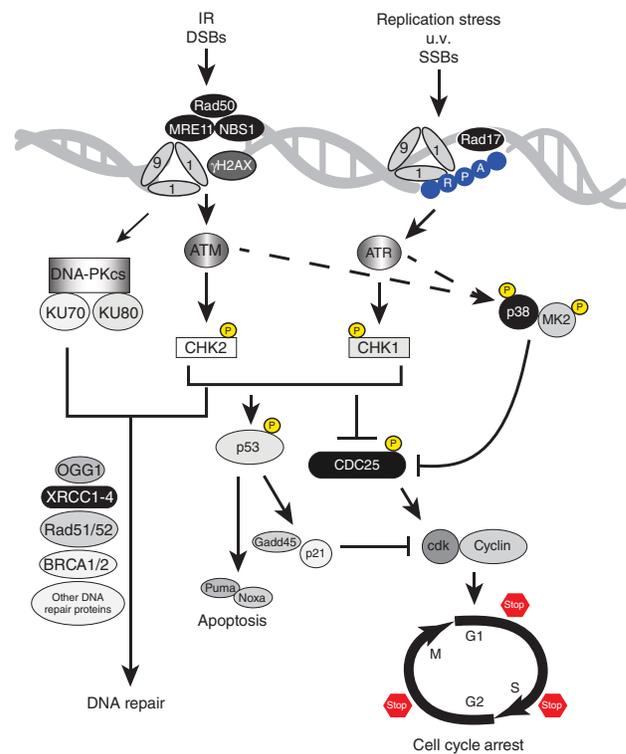

Figure 1

A simplified model of the DNA damage response. Cellular insults activate the DDR, which consists of multiple signaling pathways that ultimately lead to cell cycle arrest, DNA repair, or apoptosis. Canonically, the understanding is that the presence of DSBs triggers the ATM/CHK2 pathway and SSBs initiate the ATR/CHK1 pathway, which relies on the binding of RPA to SSBs for the activation of RAD17 and promotion of the loading of the 9-1-1 complex onto damaged DNA sites. The p38MAPK/MK2 signal transduction pathway is stimulated downstream of ATM and ATR following DNA damage. These signaling pathways modulate DNA repair through the upregulation of DNA repair proteins associated with BER, NER, MMR, HR, and NHEJ. In order to provide sufficient time to repair the DNA, these checkpoint pathways mediate the arrest of the cell cycle at the G1, S, and G2/M phases. If the damage to DNA is irreparable, p53-dependent and p53-independent apoptosis is induced. It is important to note that the DDR is not simply linear, but more complex with significant crosstalk between these signaling pathways.

Table 1 DNA repair pathways

DNA lesion	SSB	Bulky lesions crosslinks	Mismatch	DSB		
DDR pathway	BER	NER	MMR	HR	cNHEJ	aNHEJ
Integral proteins	RPA	RAD23B	**MSH2/6**	**ATM**	**ATM**	**PARP1**
	OGG1	RPA	MLH1/3	**ATR**	**ATR**	**MRN**
	APEX1/2	ERCC1-6/8	PMS1-2	**MRN**	**MRN**	CtIP
	PARP1	XPA	EXO1	**CHK1/2**	**CHK1/2**	
	PARP2	XPC	PCNA	**PARP1**	**PARP1**	
	XRCC1	XAB2	RFC	**BRCA1**	KU70/80	
	DNA polβ	PCNA	DNA polδ	**BRCA2**	**DNAPKcs**	
	PCNA	DNA polδ	Ligase I	**RAD51**	Artemis	
	FEN1	DNA polε	Ligase IV	RAD52	**XRCC4**	
	Ligase III	FEN1		p53	XLF	
		Ligase I			DNA polμ	
					Ligase IV	

Several DNA repair pathways exist to deal with various types of DNA insults. These pathways include i) BER, ii) NER, iii) MMR, iv) HR, v) cNHEJ, and vi) aNHEJ. Bold type indicates that these DDR proteins are linked to AR signaling. RPA, replication protein A; APEX, AP endonucleases; PCNA, proliferating cell number antigen; FEN1, flap endonuclease 1; ERCC, excision repair cross complementation group; XP, xenoderma pigmentosum; XAB2, XPA-binding protein 2; MSH. MutS homolog; MLH, MutL homolog; EXO1, exonuclease 1; XLF, non-homologous end-joining factor 1; CtIP,- Rb-binding protein 8.

downstream effector checkpoint kinases 1 and 2 (CHK1 and CHK2) to halt cell cycle progression, as well as recruit the DNA repair machinery to damaged sites (Table 1). SSBs or bulky DNA base adducts trigger the ATR/CHK1 signaling pathway, while DSBs primarily stimulate the ATM/CHK2 pathway. Furthermore, the coating of replication protein A (RPA) on single-stranded DNA triggers the activation of Rad17 to induce loading of the 9-1-1 complex (RAD9-HUS1-RAD1), a critical transducer of ATR/CHK1 checkpoint activation upon DNA damage (Smits et al. 2010). The stress-activated p38MAPK/MAPKAP2 (p38MAPK/MK2) pathway is a third checkpoint pathway, activated downstream of ATM and ATR in response to DNA damage (Manke et al. 2005). These signaling pathways regulate repair mechanisms through i) the transcription and post-translational modification of DNA repair genes; and ii) the modification of chromatin at the site of the DNA lesion to accommodate the recruitment of DDR factors regulating repair and signaling (Sirbu & Cortez 2013). If repair fails, checkpoint signaling initiates p53-dependent or -independent cell death programs. Therefore, these checkpoints are central nodes in the DDR network that regulate damage sensing, DNA repair, cell cycle arrest, gene transcription, and apoptosis. While these pathways are generally considered distinct entities, there is a significant overlap and cooperation between them.

To allow sufficient time to repair DNA lesions before replication or cell division, the activation of the ATR/CHK1, ATM/CHK2, and p38MAPK/MK2 checkpoint pathways delays cell cycle progression at the G1, S, and G2/M phases. p53, a major effector of these pathways, mediates the G1 arrest by upregulating the expression of growth arrest and DNA damage-inducible 45 (Gadd45) or p21/WAF1, which inhibits the G1/S-promoting cyclinE/CDK2 kinase, and subsequently, DNA synthesis (Vogelstein et al. 2000). However, if the damage to DNA is excessive, p53 initiates apoptosis through the activation of p53 target genes PUMA and NOXA (Vousden & Lu 2002). All three checkpoint pathways regulate the G1 and G2/M checkpoints through the negative modulation of the cell division cycle 25 (CDC25) phosphatase family, which are direct substrates of CHK1, CHK2, and MK2 (Reinhardt & Yaffe 2009). CDC25 proteins regulate cell cycle progression by removing the inhibitory phosphorylations on cyclin/CDK complexes. CDC25A is phosphorylated by CHK1, leading to degradation by the proteasome during the intra-S phase checkpoint. Activation of the G2/M checkpoint results in the phosphorylation of CDC25B and CDC25C by CHK1, CHK2, or MK2 and binding to 14-3-3, ultimately reducing the activity of CDC25 and causing cytoplasmic sequestration.

Several groups have proposed the hypothesis that the loss of a normal response to DNA damage may be a crucial step in the progression of PCa and polymorphisms in DDR genes that have been associated with PCa. Polymorphisms in the ATM gene are associated with an increased risk of developing PCa. Genotyping of five ATM variants in the DNA from 637 PCa patients and 445 controls with no family history of cancer revealed that there was a significant correlation between the ATM 3161G polymorphism and PCa development (Angèle et al. 2004).

DNA polymorphisms in *BER* genes are risk factors for PCa (Chen *et al.* 2001, 2003*a*, van Gils *et al.* 2002, Xu *et al.* 2002, Rybicki *et al.* 2004). PCR analysis of genomic DNA isolated from 84 patients with primary PCa and 252 individually matched controls in Caucasians showed an increased risk of PCa for patients with the Ser326Cys polymorphism of oxoguanine glycosylase 1 (*OGG1*), which is involved in excision repair of 8-hydroxy-2-deoxyguanine from oxidatively damaged DNA (Chen *et al.* 2003*a*). In addition to the *OGG1* Ser326Cys polymorphism, the *OGG1* 11 657 A/G polymorphism was discovered in a population-based and family-based association study to increase susceptibility to PCa (Xu *et al.* 2002). X-ray repair cross-complementing group 1 (*XRCC1*) polymorphisms, which reduced the capacity of *BER*, were found to increase PCa risk significantly among men consuming low vitamin E and lycopene diets (van Gils *et al.* 2002). In a large Caucasian sibling-based study examining the relationship between DNA repair capacity phenotypes and PCa susceptibility, analysis indicated that polymorphisms in *XRCC1* and xeroderma pigmentosum group D (*XPD*), which are involved in both *BER* and *NER* pathways respectively, were associated with increased PCa risk (Rybicki *et al.* 2004). These authors suggested that the reduced efficiency to repair damaged DNA may play a role in PCa, especially when two different DNA repair pathways are impaired simultaneously.

PCa cell lines have been observed to possess defective MMR pathways (Chen *et al.* 2001, Yeh *et al.* 2001). LNCaP, DU-145, DUPro, TSUPr1, and PC3 cells displayed reduced activity in DNA MMR assays, and western blotting analysis showed decreased expression of the six MMR proteins (MLH1, PMS1, PMS2, MSH2, MSH3, and MSH6). In support of this observation from cell lines, MLH1, MSH2, and PMS2 were reduced or absent in human prostate tumors (Chen *et al.* 2001, 2003*b*). However, while these reports suggest a loss of MMR in PCa, microsatellite analysis of patient samples suggests that no consensus exists regarding the loss of MMR in PCa (Dahiya *et al.* 1997, Ahman *et al.* 2001). Thus, the role of MMR in PCa progression remains an open question.

In p53-positive PCa cells, there was a significant increase in HR genes *RAD51*, *RAD52*, *RAD54*, and *XRCC3* following 10 or 25 Gy IR at both the RNA and protein levels in comparison with normal prostate epithelial cells. IR was also shown to increase the expression of *P21*, *GADD45*, *BAX*, and *BCL2* in WT p53-expressing cells (normal prostate epithelial cells, normal prostate stromal cells, and LNCaP), but not in mutant p53-expressing or p53-null cells (DU-145 and PC3 respectively) (Fan *et al.*

2004). In addition, XRCC1, a ssDNA break repair protein, was also upregulated in PCa cells treated with IR. However, neither genes making up the MRN complex nor the NHEJ genes *KU70*/80, *DNAPKCs*, *XRCC4*, and *LIGIV* were differentially expressed. Even though these data suggested that HR genes increased in malignant prostate cells after IR, alkaline and formamidopyrimidine-DNA glycosylase comet assays in PCa cells revealed that these cells were defective in the repair of SSBs, DSBs, and DNA base damage. Assays assessing chromosomal damage suggested that PCa cells acquired chromosomal aberrations, including chromatid breaks, complex radical chromosomes, and centrosome fissures. These authors presented for the first time that defects in SSBs, DSBs, and base damage repair pathways are associated with increased chromosomal aberrations and genetic instability in malignant prostate cells (Fan *et al.* 2004). The upregulation of DDR genes found in PCa may be a compensatory effect of the functional deficiency in the DDR. The specific mechanisms leading to the deficiency in DNA repair have not yet been fully elucidated, although mutations in genes regulating the DDR response have been reported (Chen *et al.* 2001, Aguilera & Gómez-González 2008, Martin *et al.* 2010, Smith *et al.* 2010).

Somatic *CHK2* mutations have been found in many cancers, including prostate, breast, colon, lung, ovary, bladder, and vulva cancers (Haruki *et al.* 2000, Miller *et al.* 2002, Reddy *et al.* 2002, Dong *et al.* 2003, Bartkova *et al.* 2004, van Puijenbroek *et al.* 2005, Nevanlinna & Bartek 2006, Williams *et al.* 2006). Germline *CHK2* alterations have been identified in familial cancers of the prostate and breast (Dong *et al.* 2003, Nevanlinna & Bartek 2006). The effect of these mutations is the characteristic of the location where the alteration is found. While aberrations in the FHA domain affect substrate recognition (Falck 2001, Li *et al.* 2002), those in the catalytic region impair kinase activity (Matsuoka *et al.* 2001, Wu *et al.* 2001). Individuals harboring certain *CHK2* mutations have an increased risk of developing prostate, colorectal, or breast cancer (Cybulski *et al.* 2004, 2007, Walsh *et al.* 2006). In addition to genetic mutations, epigenetic mechanisms have been documented to reduce CHK2 protein expression (Tort *et al.* 2002, Zhang *et al.* 2004, Williams *et al.* 2006).

Checkpoint and AR signaling in PCa

While DNA damage checkpoints are generally mediated by ATR-CHK1 and ATM-CHK2 signaling pathways (Bartek & Lukas 2003), a growing body of evidence has revealed

that the DDR extends beyond these canonical signaling pathways to include connections to AR signaling. In PCa, components of DNA damage checkpoint signaling have been regarded as AR-collaborating factors in two important processes: i) the alteration of AR activity by functioning as co-regulators and ii) the generation of chromosomal translocations (Mills 2014). The assembly of protein complexes required for DNA repair is conceptually similar to those assembly processes required for active transcription, thereby forming the basis for the idea that checkpoint signaling proteins might function as AR co-regulators. Recently, two seminal studies have established that AR signaling regulates DNA damage checkpoint signaling proteins in PCa (Goodwin et al. 2013, Polkinghorn et al. 2013).

Evidence from the last few years supports the concept that transcription involves profound changes in genome organization and structure. Exposure of cells to hormone, which generates widespread transcriptional changes, results in global alterations in genome organization. Thus, the induction of the transcriptional programs involves the recruitment of proteins involved in repair of DNA damage. Below are described some of the specific circumstances where DNA damage checkpoint signaling proteins function as AR co-regulators.

RAD9A

RAD9A, a member of the Rad family of checkpoint proteins and an integral part of the 9-1-1 complex involved in the detection of DNA damage, cell cycle arrest, and DNA repair, co-immunoprecipitated with the AR in PC3 and CWR22-Rv1 cells in an androgen-dependent manner (Wang et al. 2004). Two-hybrid and GST pull-down assays in these PCa cells showed that this interaction was mediated by the FXXLF motif at the c-terminus of RAD9A and the ligand-binding domain of the AR in the presence of dihydrotestosterone (DHT). Luciferase reporter experiments revealed that RAD9A downregulated AR transcriptional activity in PC3 and LNCaP cells by blocking the interaction between the amino- and carboxy-terminal of the AR. Furthermore, depletion of hRad9 by siRNA restored AR transcriptional activity in the presence of DHT. These effects on AR transcription were specific to the AR because RAD9A showed little or no effect on the activities of the progesterone, estrogen, or vitamin D receptors. These findings indicated that RAD9A was a novel co-repressor of the AR in PCa cells and showed how checkpoint proteins may crosstalk with AR signaling in PCa. An interesting and confounding observation is that RAD9A is highly expressed in both AR-positive and AR-negative PCa cell lines (LNCaP, CWR22, DU145, and PC3) when compared with normal prostate cells (Zhu et al. 2008). The over-expression is due to either aberrant methylation or amplification, depending on the cell line. Reducing RAD9A expression by RNAi reduced xenograft tumorigenicity. Importantly, analysis of human prostate tumor biopsies suggested that the percent of specimens with high RAD9A protein levels was elevated in tumors compared with noncancerous prostate. This is contrary to what would be predicted based on RAD9A functioning as an AR co-repressor. One plausible, but not yet explored possibility, is that RAD9A selectively represses a subset of AR-target genes. Overexpression of RAD9A may therefore contribute to the reprograming of the AR transcriptome during PCa initiation and progression.

Checkpoint kinase 1

In studying EGF regulation of the AR co-regulator melanoma antigen gene protein 11 (MAGE11), it was discovered that CHK1 phosphorylates MAGE11 on T360 in the presence of epidermal growth factor (EGF) (Bai & Wilson 2008). MAGE11 specifically binds the AR amino-terminal FXXLF motif and modulates the AR–N/C interaction to increase transcriptional activity of AR. CHK1 phosphorylation of MAGE11 T360 targets MAGE11 for ubiquitinylation at Lys240 and Lys245, which are required for the interaction of MAGE11 with the AR amino-terminal FXXLF motif. Mutation of the lysines or the T360 phosphorylation site reduced the ubiquitinylation of MAGE11 in response to EGF, ultimately leading to the loss of its co-regulator function. Moreover, the inability of a MAGE11 T360A mutant to interact with the AR and increase AR transcriptional activity indicates that the phosphorylation of MAGE11 at T360 by CHK1 is part of a mechanism whereby CHK1 regulates AR transcriptional activity.

Breast cancer 1, early onset and breast cancer 2, early onset (BRCA1 and BRCA2)

The tumor suppressor genes BRCA1 and BRCA2 are AR co-activators. BRCA1, a nuclear phospho-protein that possesses E3 ubiquitin ligase activity with roles in DNA repair, mitotic cell cycle control, and transcriptional regulation, interacted with the AR amino-terminal domain in glutathione S-transferase (GST) pull-down experiments in PCa cells, and chloramphenicol acetyl-transferase (CAT) reporter assays revealed that BRCA1

enhanced transcription of AR-target genes (Park *et al.* 2000, Yeh *et al.* 2000). Consistent with BRCA1 functioning as an AR co-activator, BRCA1 protein expression was elevated in primary PCa specimens when compared with noncancerous prostate samples (Schayek *et al.* 2009). In the context of the AR-negative PC3 cells, where exogenous expression of the AR induces cell differentiation (Litvinov *et al.* 2006), ectopically expressed BRCA1 in AR-expressing PC3 cells cooperated with the AR to induce cell death in the presence of DHT, as determined by the thiazolyl blue (MTT) and propidium iodide (PI) exclusion assays (Park *et al.* 2000, Yeh *et al.* 2000). These authors suggested that BRCA1 played an important role in androgen-induced apoptosis by upregulating the expression of p21, since DHT dramatically induced the expression of p21 in PCa cells expressing BRCA1 and the AR (Yeh *et al.* 2000).

BRCA2, an integral component of the HR machinery, was shown to associate with the AR and enhance the transcriptional activity of the AR (Shin & Verma 2003). Co-expression of BRCA2 and AR increased luciferase reporter gene activity only in the presence of DHT, and BRCA2 expression alone had no discernible effect with hormone. Co-immunoprecipitation of mutant forms of AR revealed that BRCA2 interacted with the amino-terminal domain containing activation function 1 (AF1) and the carboxyl-terminal domain harboring AF2, suggesting that BRCA2 may be co-activators of AF1 and AF2 functions. The physiologic conditions under which BRCA1 and BRCA2 regulate AR transcriptional activity are still unknown. Men with germline *BRCA* mutations have elevated risk for PCa (Li *et al.* 2013, Bancroft *et al.* 2014). How these mutations affect AR transactivation have not yet been studied. Recently reports have emerged implicating the AR in breast cancer (Hickey *et al.* 2012). To date, the role of *BRCA* mutations on AR regulation in breast cancer have not been reported.

DNA-dependent protein kinase

The heterotrimeric DNA–PK complex, known for its role in DNA repair, has emerged as part of the AR transcriptional machinery (Mayeur *et al.* 2005; Fig. 2A). Immunoprecipitation of the AR from LNCaP cells followed by tandem mass spectroscopy showed that the AR ligand-binding domain interacted directly with the KU70 and KU80 regulatory subunits of DNA-PK in the cytoplasm and nucleus in a DNA-independent manner. Evidence suggests that this interaction is modulated by AR phosphorylation on S578 and EGFR signaling because a S578A mutant binds KU70/80 more effectively than WT AR (Ponguta *et al.* 2008).
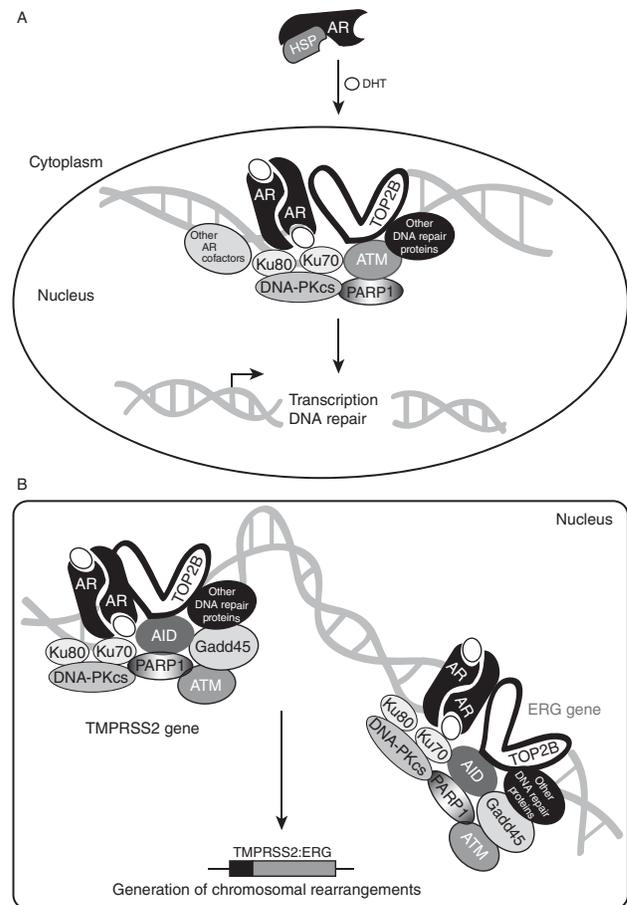

Figure 2

The convergence of DNA damage checkpoint and AR signaling pathways in prostate cancer. (A) DNA damage proteins and AR cooperate during gene transcription and DNA repair. In addition to its role in DNA repair, DNA-PK interacts with the AR in the cytoplasm and nucleus and regulates the transcriptional activity of the AR. PARP1 affects prostate cancer cell growth and progression through the modulation of AR transcriptional activity. TOP2B is required for AR-mediated transcription by facilitating transient DSBs. (B) The generation of gene rearrangements is mediated by DNA damage and AR transactivation. DHT induces the recruitment of *TOP2B*, *AID*, and *GADD45* to the promoter and enhancer of AR-regulated genes where it cooperates with the AR to generate gene translocations such as TMPRSS2:ERG.

Knockdown of Ku proteins by siRNA diminished AR transcriptional activity, as measured by a PSA promoter luciferase reporter (Mayeur *et al.* 2005). Furthermore, expression of DNA-PKcs enhanced AR transcriptional activity for two individual AR reporter constructs. Chromatin immunoprecipitation (ChIP) experiments in LNCaP cells showed that Ku proteins were recruited in an androgen-dependent manner to the promoter of AR-target genes. However, the S578A AR phosphorylation site mutant with enhanced KU70/80 binding had significantly reduced AR transcriptional activity (Ponguta *et al.* 2008).

Thus, enhanced KU70/80 binding is insufficient for AR transactivation. These data suggest that KU70, KU80, and DNA-PKcs are AR transcriptional regulators; the biological context of which remains to be fully elucidated.

The interaction between the AR and KU70 was also shown to exist *in vivo*, as co-immunoprecipitation of AR from tissue samples of PCa patients before and after castration indicated that the AR and KU70 associated in pre- and post-castration extracts (Al-Ubaidi *et al.* 2013). Furthermore, IHC revealed that castration resulted in a decrease in the expression of KU70, which correlated with the decline in PSA levels. The castration-induced loss of KU70 protein was also associated with an increase in γH2AX foci formation. These data indicated that the decrease in KU70 protein associated with decreased AR activity impaired the repair of DNA damage.

Poly (ADP-ribose) polymerase 1

PARP1, an enzyme that coordinates the assembly and activation of the BER machinery of the DDR (El-Khamisy *et al.* 2003) and recently implicated in the emerging aNHEJ pathway (Boboila *et al.* 2012), has recently been determined to regulate PCa cell growth and progression through the modulation of AR transcriptional activity (Schiewer *et al.* 2012; Fig. 2A). The PARP inhibitor ABT888 sensitized androgen deprivation therapy (ADT)-sensitive and castration-resistant PCa (CRPC) cells to IR and docetaxel, as the number of ADT-sensitive cells decreased in response to ABT888 alone and decreased further in combination with radiation or chemotherapy in trypan blue exclusion assays. AR-negative PCa cells were not affected by ABT888, suggesting that the AR mediated the biological effects of PARP1 inhibition. Importantly, PARP1 inhibition had no effect on AR-positive normal prostate cells. The inhibition of PARP1 activity reduced the transcription of clinically relevant prostate-specific AR-target genes, indicating that PARP1 is a critical effector of AR activity. Remarkably, ChIP–qPCR revealed that both PARP1 and the AR were recruited to the enhancer and promoter of AR-target genes in response to DHT, and the addition of ABT888 suppressed this recruitment, thereby suggesting that PARP1 activity may be necessary for the mobilization of AR and PARP1 to the key sites of AR function. Furthermore, while the nonsteroidal AR inhibitor Casodex was ineffective at suppressing AR activity in CRPC cells, PARP inhibition with ABT888 or shRNAs decreased the transcription of AR-target genes. Using human tumor xenografts, the *in vitro* effects of PARP1 inhibition on growth and AR transcriptional activity were

recapitulated *in vivo*. Interestingly, PARP1 also regulates PCa cell growth and invasion through interactions with the *ETS* gene fusion product, ERG (Brenner *et al.* 2011). Inhibition of PARP1 blocks ERG-driven transcription, cell invasion, and *in vivo* xenograft growth. These data establish that PARP1 is not only a critical member of the DDR, but also a robust mediator of two major drivers of PCa: *AR* and *ETS* gene fusion products. The relative requirement of PARP1 – AR or PARP1 – ERG interactions for the biological effect of PARP1 inhibition is presently unknown.

Topoisomerase IIB and the generation of chromosomal rearrangements

The kinase activity of topoisomerase IIB (TOP2B), an enzyme that catalyzes transient DSBs to resolve DNA topological constraints, was required for the induction of AR-mediated transcription (Ju *et al.* 2006; Fig. 2A). DNA break-labeling ChIP assays showed that there was an increase in biotin incorporation in the promoter of the prostate-specific antigen (*PSA*) gene following DHT treatment. Inhibition of TOP2B prevented AR-dependent transcriptional activation. These data suggest that TOP2B mediates transient DSBs at the endogenous promoter of AR-target genes, and that DNA repair proteins play crucial roles in AR-transcriptional regulation.

In addition to playing a direct role in AR-mediated transcription, TOP2B has been hypothesized to be involved in the generation of *TMPRSS2–ERG* rearrangements with the assistance of AR signaling (Haffner *et al.* 2010; Fig. 2B). Gene expression microarray analysis followed by RT-PCR showed that the inhibition of TOP2B with chemical agents or shRNAs in LNCaP or LAPC4 cells decreased the upregulation of DHT-induced genes. AR and TOP2B were recruited to the enhancer and promoter of androgen-regulated genes in DHT-treated LNCaP and LAPC4 cells as a complex. Potassium SDS (KSDS) assays, which allowed the isolation of etoposide-stabilized TOP2B catalytic cleavage complexes, showed that cleavage at the enhancer and promoter of androgen-regulated genes in the presence of DHT was mediated by TOP2B, as knockdown of TOP2B abolished cleavage. Furthermore, these KSDS experiments revealed breakpoint hotspots and regions with high levels of DHT-induced TOP2B cleavage that were enriched for proximity to TMPRSS2 and ERG breakpoints. DHT induced the recruitment of AR and TOP2B to these cleavage sites in spite of the absence of AREs. It is important to note that the AR binds to thousands of genomic DNA sites that lack AREs

(Wang *et al.* 2007). Positive staining for H2AX phosphorylation and ATM suggested that DHT induced DSBs at sites where the AR and TOP2B were recruited (Schiewer *et al.* 2012). Using dual-color fluorescence *in situ* hybridization in LNCaP and LAPC4 cells, DHT increased genomic breaks in TMPRSS2, and etoposide enhanced the effects of DHT, suggesting that active AR may cooperate with TOP2B to induce genomic breaks and/or chromosomal translocations at these loci (Haffner *et al.* 2010). These data indicate a model where intrinsic AR signaling in PCa cells induces TOP2B-mediated DSBs at many genomic loci, even without genotoxic stress. These DSBs may thus participate in genomic recombination events and produce rare rearrangements.

In support of this concept, transient androgen exposure was shown to promote the induction of translocations in PCa cells, but not in nonmalignant prostate epithelial cells (Bastus *et al.* 2010). However, prolonged androgen treatment induced TMPRSS2:ERG fusions in nonmalignant prostate epithelial cells. These data suggested that the presence of functional DDR components prevented genomic instability, which were mutated in PCa cells. The ATM and ATR DNA damage checkpoints were activated by androgen, as phosphorylation of ATM, ATR, CHK1, CHK2, and H2AX was upregulated upon androgen exposure in nonmalignant prostate epithelial cells stably expressing AR (Chiu *et al.* 2012). Inactivation of the ATM or ATR checkpoints with siRNA in the presence of androgen suppressed the phosphorylation of H2AX and resulted in the generation of fusion transcripts, indicating that the ATM and ATR DNA damage checkpoints acted as a surveillance system to guard against androgen-induced chromosomal translocations. Androgen induced the activation of ATM in LNCaP cells, but decreased the phosphorylation state of ATR, CHK1, CHK2, and H2AX, suggesting that the ATM pathway was partially impaired in LNCaP cells. These data indicated that the ATM and ATR checkpoints suppressed chromosomal translocations induced by androgen in prostate cells, and the inactivation of these pathways may facilitate genomic instability promoted by androgen and thereby contribute to prostate tumorigenesis.

In addition to TOP2B, the AR collaborated with both activation-induced cytidine deaminase (AID) and GADD45 in LNCaP cells treated with DHT and IR to facilitate chromosomal translocations (Lin *et al.* 2009). AID associates with DNA-PKcs and promotes cell survival through the resolution of DNA DSBs (Wu *et al.* 2005); GADD45 proteins have important roles in DNA repair mechanisms by modulating the NER, BER, and MMR machinery through its interaction with proliferating cell nuclear antigen (PCNA) (Tamura *et al.* 2012). The AR-AID-GADD45 interaction was dependent on DHT, as IR alone did not promote this association. Casodex repressed the recruitment of AID and GADD45 to AR binding sites, suggesting that the activity of the AR was required for the association with AID and GADD45. ChIP analyses revealed that AID was recruited to intronic AR-binding sites on *TMPRSS2*, *ETV1*, and *ERG* translocations regions, as well as AR-target genes, in response to DHT and IR. Depletion of AID from LNCaP cells suggested that AID may be essential for the generation of DSBs at translocation regions, as ChIP experiments-revealed decreased Ku80 enrichment and BrdU/TdT assays confirmed the ChIP results.

The concept that the androgen-induced transcriptional program promotes DSBs, and that the DNA repair machinery is required to maintain genomic integrity during this process, has profound implications for PCa therapy. A major mechanism of cancer chemotherapeutics is the generation of DSBs leading to the potentiality of exploiting the DSBs generated by androgen-regulated transcription. This raises the exciting possibility that cycling hormone treatment and ablation in CRPC in combination with chemotherapeutics that induce DSBs or new inhibitors of the DNA repair machinery could lead to more efficacious treatments (Haffner *et al.* 2011). However, not surprisingly, there are confounding variables to this idea, which the research described below illustrate.

AR signaling regulates the DDR in response to cellular insults

The activation of the AR augments the induction of the DDR and cell death in PCa cells exposed to oxidative stress (Ide *et al.* 2012). In LNCaP and Rv1 cells treated with synthetic androgen (R1881) and hydrogen peroxide (H_2O_2), ATM and CHK2 phosphorylation, as well as PARP cleavage, was elevated in comparison to cells treated with H_2O_2 alone. The AR inhibitor flutamide and ATM inhibitor Ku55933 both reduced PARP cleavage induced by R1881 and H_2O_2 in both cell lines. Similar effects on ATM, CHK2, and PARP were seen with DHT. These data suggest that AR signaling may cooperate with DDR signal transduction to suppress prostate carcinogenesis and malignant transformation.

AR activity also positively regulated cell death induced by DNA damage from taxane-based cytotoxic insult (Hess-Wilson *et al.* 2006). While androgen had no effect on paclitaxel-induced cell death in PC3 cells, LNCaP cells cultured with mitogenic levels of hormone exhibited

reduced cell number when exposed to paclitaxel in trypan blue exclusion assays. However, androgen did not sensitize castration resistant or AR-negative PCa cells, suggesting that the hormone environment sensitized AR-dependent PCa cells to paclitaxel. In AR-positive androgen-dependent cells, the anti-androgen Casodex reversed the effect of hormone and paclitaxel. Similar results were seen with docetaxel, thus implicating AR activity in the synergism with taxanes to enhance cell death.

In contrast to taxanes, AR activity antagonized topoisomerase inhibitors and platinum drugs, as the efficacy of these drugs appears to rely on the inhibition of AR activity (Mantoni *et al.* 2006). The chemotherapeutic drugs etoposide and cisplatin inhibited the activity of the AR on luciferase reporter constructs in a dose-dependent manner in LNCaP and PC3 cells expressing exogenous AR. These drugs did not significantly affect the cell cycle, expression of the AR, or prevent AR nuclear translocation in response to DHT, suggesting that there is another mechanism for the loss in AR activity. ChIP studies showed that etoposide and cisplatin reduced the recruitment of AR to the PSA enhancer, indicating that the decreased recruitment of AR to AREs may be a mechanism by which AR inhibition acts in concert with therapeutic agents that cause DNA damage.

AR signaling was recently demonstrated to be a key regulator of the DDR in two provocative studies (Goodwin *et al.* 2013, Polkinghorn *et al.* 2013). Goodwin *et al.* established that ADT (charcoal striped serum) and IR suppressed doubling times and survival of LNCaP and LAPC4 cells when compared with IR alone. However, the combination treatment had no significant effect on cell number in AR-negative PC3 cells, suggesting that AR blockade in androgen-sensitive cells enhanced the response to radiation *in vitro*. Xenograft tumor growth experiments with C42 cells revealed that the combination of ADT and IR was more effective than either treatment alone at suppressing growth and enhancing survival, indicating that ADT cooperated with radiation to decrease tumor growth and survival in ADT-sensitive, AR-positive PCa cells. Consistent with this experimental result, ADT is the only clinically confirmed sensitizer to radiation therapy leading to improved survival (Bolla *et al.* 2010, Koontz & Lee 2011). The suppression of growth induced by ADT and radiation was rescued with resupplementation of DHT, suggesting that androgen and the AR promoted resistance to genotoxic insult (Goodwin *et al.* 2013). The effect of androgen and AR on radiation responses was independent of AR-dependent cell cycle control, as the combination treatment dramatically suppressed growth in

comparison with ADT or radiation alone in castration-resistant C42 and Rv1 cells, which are insensitive to the ADT-induced G1 arrest. The anti-androgen MDV3100 showed similar results as those seen with ADT. These data show that active AR promoted resistance to radiation.

As AR promoted radiation resistance independently of alterations in cell cycle control, the effect of AR on DNA repair was examined. γH2AX and 53BP1 foci determination showed that ADT impaired DSB repair, and DHT resupplementation rescued this defect. While DHT decreased DNA fragmentation in comet assays, ADT and radiation elevated fragmentation. These data suggest that the AR promotes DNA DSB repair, independent of the effect of the AR on the regulation of cell cycle progression.

Using a custom qPCR array to detect changes in the DNA damage and repair-associated transcriptome after radiation, PRKDC (DNA-PKcs), XRCC2, and XRCC3 were identified as AR-occupied regions, suggesting that the AR regulated the expression of DNA damage repair genes. Furthermore, western blotting showed that CRPC cells treated with DHT elevated DNA-PKcs expression and activity in the presence and absence of IR. γH2AX and 53BP1 foci assessment in DNA-PKcs RNAi-expressing cells determined that AR-mediated DSB repair and growth following radiation required DNA-PKcs expression and activity. When DNA-PKcs activity is inhibited, repair of IR-induced DNA lesions by NHEJ is weakly increased with AR activation, suggesting that DNA-PKcs function is required for AR-mediated DNA repair. These results further support previous studies showing that DNA-PK interacted with the AR and functioned as an AR co-activator under certain conditions (Mayeur *et al.* 2005). Collectively, the research identified a critical feedback loop between hormone action and DNA repair, where the regulation of DNA-PKcs expression and activity by AR is required for AR-mediated DNA repair and therapeutic resistance.

The repertoire of AR-regulated *DDR* genes was expanded in a study reported by Polkinghorn *et al.* (2013), who discovered that the AR modulated the transcription of a network of DNA damage repair genes in CRPC. Transcriptome and gene-set enrichment analyses on CRPC cells treated with the second-generation anti-androgen ARN-509 for 4 days surprisingly showed that DNA repair gene sets were represented in three out of the top ten gene sets enriched in the control vs the ARN-509-treated groups. Furthermore, there was a significant correlation between AR transcriptional output and the enriched *DDR* genes in primary prostate tumors. An 'AR-associated DNA repair gene' signature of 144 DNA repair genes that were significantly associated with canonical AR

output was defined. Using RNA-seq followed by ChIP-seq, 32 of the 144 'AR-associated DNA repair genes' were validated as true AR-target genes in LNCaP cells treated with or without synthetic androgen for 2 days. Upon exposing these same LNCaP cells to IR, γH2AX foci formed later and DSBs resolved more slowly under androgen-depleted conditions, which was consistent with impaired DNA repair. These data were recapitulated by the Comet assay, which directly measured DNA DSBs. Even in the presence of ARN-509 alone, LNCaP cells, as well as VCaP cells and the CRPC cell line LNCaP-AR showed increased DNA damage and cell death, as cells treated with ARN-509 exhibited more DNA tails in Comet assays and decreased survival in clonogenic survival experiments. While V(D)J recombination analyses revealed that anti-androgen treatment dramatically decreased DNA repair by inhibiting NHEJ, DNA repair reporter experiments showed that ARN-509 had no significant effects on HR. Together, these data demonstrated that AR signaling regulated a network of DNA damage repair genes that included members of DNA damage sensors (ATR, MRE11A), NHEJ (XRCC4, XRCC5), HR (RAD51C, Rad54B), BER (PARP1, LIG3), and MMR (MSH2, MSH6). Furthermore, AR activation increased the transcription of DNA repair genes, and concurrently promoted resistance to radiation through the rapid repair of IR-induced DNA damage.

These results linking the AR to the regulation of the DDR confound how to leverage the androgen-induced DSBs discovered as part of the androgen-induced transcriptional program. Furthermore, it highlights the general deficiencies in our understanding of how AR functions and integrates with the DDR, despite all that we currently know.

Conclusion

PCa is a unique malignancy in that it still depends on AR signaling for development, growth, and survival. The DDR acts as a barrier to tumorigenesis by preventing the accumulation of genome instability. The link between the DDR machinery and PCa has been well documented, as defects in the normal response to DNA damage can either predispose to (through the acquisition of mutations due to the inability to repair DNA lesions) or facilitate progression of PCa. Until recently, the signaling pathways governing the DDR have been considered to be composed of two canonical signal transduction pathways: ATR/CHK1 and ATM/CHK2. In the past 10 years, significant evidence has implicated critical connections between DDR pathways and AR signaling in PCa. The DDR machinery and AR have been linked in the regulation of AR transcriptional activity, chromosomal translocations, DDR activation and gene expression, and therapeutic resistance. However, the biological context is critical for these linkages. While DDR and AR crosstalk provides a mechanism for the synergism seen with, and validates the rational for, the combinatorial therapy of ADT and radiation, it also raises important concerns to contemplate for future PCa treatment modalities, especially when DNA damaging drugs are being considered. In addition to those raised above, several questions still remain to be answered: i) which *DDR* genes upregulated by AR signaling following IR are crucial for growth, survival, and/or therapeutic resistance; ii) what role IR-induced oxidative stress plays in the regulation of *DDR* gene expression mediated by AR signaling; iii) which subset of cells is regulated by these connections given the heterogeneity of PCa; iv) can we use AR transcriptional activity, i.e. expression of *DDR* genes, to identify patients that will benefit from ADT and/or radiation therapy; v) what additional drug(s) can be combined with ADT to decrease mortality of CRPC; vi) and finally, can we extend this knowledge to other endocrine-related cancers, such as breast and ovarian cancers.

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