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Deciphering the role of distinct DNA-PK phosphorylations at collapsed replication forks.

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Abstract

It has recently been established that the marked sensitivity of ATM deficient cells to topoisomerase poisons like camptothecin (Cpt) results from unrestrained end-joining of DNA ends at collapsed replication forks that is mediated by the non-homologous end joining [NHEJ] pathway and results in the induction of copious numbers of genomic alterations, termed “toxic NHEJ”. Ablation of core components of the NHEJ pathway reverses the Cpt sensitivity of ATM deficient cells, but inhibition of DNA-PKcs does not. Here, we show that complete ablation of DNA-PKcs partially reverses the Cpt sensitivity of ATM deficient cells; thus, ATM deficient cells lacking DNA-PKcs are more resistant to Cpt than cells expressing DNA-PKcs. However, the relative sensitivity of DNA-PKcs proficient ATM deficient cells is inversely proportional to DNA-PKcs expression levels. These data suggest that DNA-PK may phosphorylate an ATM target (that contributes to Cpt resistance), explaining partial rescue of Cpt sensitivity in cells expressing high levels of DNA-PKcs.

Although crippling NHEJ function by mutagenic blockade of the critical ABCDE autophosphorylation sites in DNA-PKcs also sensitizes cells to Cpt, this sensitization apparently occurs by a distinct mechanism from ATM ablation because blockade of these sites actually rescues ATM deficient cells from toxic NHEJ. These data are consistent with autophosphorylation of the ABCDE sites (and not ATM mediated phosphorylation) in response to Cpt-induced damage. In contrast, blockade of S3205 (an ATM dependent phosphorylation site in DNA-PKcs) that minimally impacts NHEJ, increases Cpt sensitivity. In sum, these data suggest that ATM and DNA-PK cooperate to facilitate Cpt-induced DNA damage, and that ATM phosphorylation of S3205 facilitates appropriate repair at collapsed replication forks.

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Dr. Meek and Dr. Neal were responsible for conceptualization of experiments and development of all of the cell culture models used in the manuscript.

All four authors performed experiments and prepared graphical representations of experiments.

Dr. Meek was responsible for manuscript preparation.

Dr. Meek's funding sources provided research resources for the experiments presented in the manuscript.

Dr. Meek is responsible for oversight and all leadership responsibilities required for execution of experiments and development of the manuscript.

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Keywords

Non-homologous end joining (NHEJ); DNA dependent protein kinase (DNA-PK); DNA-PK catalytic subunit (DNA-PKcs); toxic NHEJ

Introduction

Balmus et al. recently reported that the marked sensitivity of ATM deficient cells to the topoisomerase I poison, camptothecin (Cpt) could be markedly attenuated by ablation of most core non-homologous end joining (NHEJ) factors (Ku, XRCC4, LigIV, XLF) but not by chemical inhibition of DNA-PKcs [1]. These authors concluded that hypersensitivity of ATM deficient cells was the result of NHEJ mediated joining of single DNA ends (which the authors termed “toxic NHEJ”) that result from replication fork collapse; toxic NHEJ required core end-joining factors but was independent of DNA-PKcs. They proposed a model whereby ATM phosphorylation [of an unknown target(s)] blocks NHEJ at Cpt-induced single DNA ends at collapsed replication forks (Fig. 1A); Ctip is also required for repair of Cpt-induced damage, but Ctip’s impact is completely epistatic with ATM’s [1, 2]. Moreover, loss of Ctip induces much less Cpt sensitivity suggesting that ATM’s inhibition of toxic NHEJ involves other ATM targets. In addition, it has previously been shown that Ctip and MRN cooperate in an ATM dependent manner to facilitate removal of Ku at single DNA ends at collapsed replication forks [2, 3], although persistence of Ku did not directly correlate with toxic NHEJ in the Balmus et al study. Finally, emerging data suggest that DNA-PK may facilitate MRN-dependent end-processing at single DNA ends [4]. Still, precisely how ATM inhibits toxic NHEJ is not known.

In the Balmus et al. study, no ATM/DNA-PKcs doubly deficient cell strains were utilized. We have previously generated a number of CHO cell strains that lack both DNA-PKcs and ATM, and we utilized these cell strains to assess the impact of DNA-PKcs on toxic NHEJ in ATM deficient cells [5]. Our studies demonstrate that DNA-PKcs also contributes to toxic NHEJ.

We have previously characterized numerous DNA-PKcs auto-phosphorylation sites [6–10]. Phosphorylation of two major clusters (ABCDE and PQR) reciprocally regulate end processing during repair of two-ended DSBs. Blocking the ABCDE cluster impairs end processing, consistent with the finding that ABCDE phosphorylation is required to activate the Artemis nuclease [11–13]. In contrast, blocking the PQR cluster promotes end processing. Like ablation of ATM, blocking the ABCDE cluster induces marked cellular sensitivity to camptothecin; however, ablation of ABCDE phosphorylation also results in cellular sensitivity to many DNA damaging agents including agents that induce DSBs (IR, zeocin, bleomycin, neocarzinostatin) as well as DNA damaging agents that causes replication-associated damage (cisplatin, UV, MMS, and, HU) [10]. With all of these DNA damaging agents, cells expressing DNA-PKcs that cannot phosphorylate the ABCDE cluster are more sensitive to DNA damage than are cells lacking DNA-PKcs, suggestive of a dominant negative cellular phenotype. We (and others) have addressed the mechanistic basis for the hypersensitivity of cells expressing DNA-PKcs that cannot phosphorylate the

ABCDE sites. We have shown that the kinetics of repair in these cells (as measured by resolution of IR-induced γ H2AX foci) is delayed [7]; this is similar to the observation of delayed clearance of drug-DNA adducts after exposure to cisplatin [10]. Moreover, blocking ABCDE phosphorylation impairs release of DNA-PKcs both *in vitro* and in living cells [8, 14]. The increased occupancy of DNA-PKcs at DSBs impairs sister chromatic exchange and impacts cellular HR assays [15–18]. But even though cellular sensitivity is greatly increased in cells that cannot phosphorylate the ABCDE sites as compared to cells that lack DNA-PKcs, the level of NHEJ measured in these cells (by assessing episomal VDJ recombination) although reduced compared to wild type cells, is actually more efficient than in cells lacking DNA-PKcs [10]. Because of these observations suggesting that blocking ABCDE phosphorylation actually impedes (but does not completely block) repair (and to differentiate the basis of this cellular sensitivity to Cpt from the “toxic NHEJ” that is observed after ATM ablation), we will refer to the dominant negative-like effect of blocking ABCDE phosphorylation as “slow NHEJ”.

Mechanistically, we have shown that blocking ABCDE autophosphorylation is not equivalent to blocking DNA-PK’s catalytic activity. In fact, the “slow NHEJ” effect of ABCDE-blocked DNA-PKcs at two ended DSBs requires both DNA-PK’s catalytic activity and phosphorylation of the PQR (and other) DNA-PKcs sites [10], suggesting a model whereby ABCDE autophosphorylation both regulates Artemis dependent end-processing and facilitates kinase dissociation, whereas PQR autophosphorylation facilitates stability of the DNA-PK end-bound complex. Thus, DNA-PK shelters DNA ends when ABCDE sites are blocked, but this requires phosphorylation of PQR sites that are proximate to the DNA binding pocket [19]. Finally, we show here that blocking ABCDE phosphorylation and the resultant “slow NHEJ” protects ATM deficient cells from Cpt-induced toxic NHEJ. These data are consistent with autophosphorylation of the ABCDE sites (and not ATM mediated phosphorylation) in response to Cpt-induced damage. In contrast, blockade of S3205 (an ATM dependent phosphorylation site in DNA-PKcs) that minimally impacts NHEJ, increases Cpt sensitivity, suggesting that ATM phosphorylation of S3205 restrains NHEJ at collapsed replication forks.

Materials and Methods

Plasmids, cell culture and cell strains.

DNA-PKcs expression plasmids for wild type, phospho-blocking, kinase inactivating mutants, as well as combination mutants have been described previously [10, 20]. Methods to derive V3 transfectants have been previously described [17, 20]. Briefly, the parental cell strain was co-transfected with the indicated DNA-PKcs expression plasmid and the pSuper-puro plasmid, to confer puromycin resistance, using FuGENE 6 (Roche) according to the manufacturer’s instructions. Independent, stable transfectants were selected and maintained in complete medium containing 10 μ g/ml puromycin. At least two independently derived clones expressing each mutant were studied; the parental V3 clones used in this study were extensively characterized in our previous studies [10, 17]. Stable V3 transfectants and XR-1 cells were cultured in alpha-MEM with 10% fetal bovine serum (Atlanta Biologicals, GA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml

penicillin, 100 µg/ml streptomycin (Life Technologies) and 10 µg/ml ciprofloxacin. 293T cells were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml ciprofloxacin. The ATM deficient 293T clone has been described and characterized in previous publications [5, 21]. Cells were maintained at 37°C with 5% CO₂.

Cas9 mediated gene disruption.

Cas9 targeted gene disruption of ATM and DNA-PKcs was performed using methods similar to that reported by Mali et al. [22], and adapted by our laboratory previously, designing gRNAs that work in both hamster and human cells strains [5]. Briefly, gRNAs specific for ATM or DNA-PKcs were synthesized as 455bp fragments (Integrated DNA technologies). The synthesized fragment were cloned into pCR2.1 using a TOPO TA cloning kit according to the manufacturers' instructions (Life Technologies). Cells were transfected with 1 µg gRNA plasmid and 1 µg Cas9 expression plasmid (Addgene). In some cases, cells were co-transfected with 0.2 µg of pcDNA6 (Life Technologies) to confer blasticidin resistance. Western blotting was used to identify clones with ATM ablation; in all cases, deletion was also confirmed by PCR amplification and sequencing that revealed deletions and frame shifts at the target site. The sequence of the 19mer specific for ATM synthesized into the 455bp fragments was: TCTTTCTGTGAGAAAATAC. The sequence of the 19mer specific for DNA-PKcs synthesized into the 455bp fragments was: TGCAACTTCACTAAGTCCA.

Survival assays.

Clonogenic survival assays were performed for V3 and XR-1 cells. Briefly, a hundred cells were plated for each transfectant into complete medium containing the indicated dose of zeocin, camptothecin, or calicheamicin in 60 mm diameter tissue culture dishes. After 7 to 10 days, cell colonies were stained with 1% (w/v) crystal violet in ethanol to measure relative survival. MTT staining was performed to assess cell viability in figures 1D, 4, 5, and supplemental Fig.1. 30,000 to 50,000 cells were plated in each well of a 24-well plate, containing medium with varying concentrations of camptothecin. After 5 to 7 days, cells were treated with 1 mg/ml MTT (Sigma) solution for 1 hr. Medium containing MTT was then removed and formazan crystals thus produced were solubilized in acidic isopropanol. Absorbance was read at 570 nm to determine relative survival. Data from a minimum of four independent experiments is presented in each figure. Error bars depict standard error of the mean.

Immunoblot Analyses.

Immunoblotting was performed as described [23]. Antibodies used in this study are rabbit polyclonal anti-XRCC4 (Abcam, 213729), rabbit anti-ATM (Abcam, 2C1), goat anti-lamin-B (Santa Cruz, sc621), mouse anti-γH2AX (Millipore, 05-636), mouse anti-B-actin (AC-15, Sigma), and rabbit anti-53BP1 (Abcam, 36823). The DNA-PKcs antibody (mouse monoclonal 42-27) was the generous gift of Tim Carter. DNA-PKcs phospho-specific antibodies utilized in this study include anti-phospho-S2056 (Abcam 18192, Abcam 124918), and a rabbit anti-phospho-T2609 reagent, a generous gift of Dale Ramsden.

Results

DNA-PKcs contributes to toxic NHEJ; but increasing levels of DNA-PK promote Cpt resistance in ATM deficient NHEJ proficient cells in a titratable manner.

We have recently utilized Crispr/Cas9 strategies to disrupt ATM in V3 cells to interrogate how DNA-PK and ATM affect the RAG post-cleavage complex during VDJ recombination [5, 24]. In these studies, ATM was disrupted in two V3 transfectants: 1) a wild type DNA-PKcs complemented transfectant, and 2) a vector control transfectant. Numerous ATM deficient subclones were isolated. [Supplemental Table 1 summarizes the derivation of all of the CHO cell strains utilized in this study. The parental V3 transfectants were characterized in our previous study [10].] We observed a wide range of DNA-PKcs expression in the resultant clones (Fig. 1B). [It is not unusual to observe changes in transgene expression in subclones of CHO transfectants, unpublished observations, K. Meek.] Zeocin sensitivity was assessed using clonogenic survival assays on these ATM deficient clones, testing two clones expressing very high levels of DNA-PKcs and two clones expressing minimal levels. As expected, the ATM proficient wild type DNA-PKcs expressing parental clone is more zeocin resistant than the vector control cell strain; moreover, ATM ablation exacerbates cellular zeocin sensitivity in both DNA-PK proficient and deficient V3 cells (Fig. 1C). However, variation in DNA-PKcs expression does not affect the relative zeocin resistance in DNA-PKcs complemented cells.

Balmus et al. proposed that ATM inhibits toxic NHEJ at single DNA ends induced by collapsed replication forks (Fig. 1A); as reported by Balmus et al, ablation of ATM substantially sensitized cells to Cpt; this is true both in V3 cells that have been complemented with DNA-PKcs and those transfected with vector alone (Fig. 1D). The ATM deficient vector control clones were more Cpt-resistant than any of the NHEJ proficient, DNA-PKcs expressing cell clones. [Only one clone is presented in Fig. 1; 3 additional ATM deficient vector only clones were tested and all displayed similar Cpt sensitivity (Sup. Fig. 1).] In contrast, the ATM deficient, DNA-PKcs complemented clones show a large variability in Cpt sensitivity, but are in general, more Cpt sensitive than ATM deficient cells lacking DNA-PKcs. This marked sensitivity to Cpt in ATM deficient, DNA-PKcs proficient cells can be markedly reversed by stable introduction of human ATM (Sup. Fig.2); partial complementation may reflect a requirement for hamster versus human ATM, dysregulated and/or slightly lower expression of the ATM transgene, or off-target effects of CRISPR-mediated ATM ablation. Still, the impact of ATM ablation in numerous independent clones from these two cell strains is both internally consistent and consistent with Balmus et al.

Of note, in the ATM deficient, DNA-PKcs proficient clones, those with higher levels of DNA-PKcs were increasingly more resistant to Cpt. The clone with the highest DNA-PKcs expression (WT/AT-/-42) is similarly Cpt resistant as cells lacking DNA-PKcs; whereas clone WT/AT-/-17 with minimal DNA-PKcs is exquisitely sensitive to Cpt. To explain these results, we suggest that DNA-PKcs contributes to toxic NHEJ in ATM deficient cells, explaining increased sensitivity of DNA-PKcs expressing clones as compared to ATM deficient vector only controls. The observation that the NHEJ proficient ATM deficient clones with higher levels of DNA-PKcs are more Cpt resistant, suggests that DNA-PK may

phosphorylate a target preferentially phosphorylated by ATM that facilitates Cpt resistance. Thus, although clones WT/AT^{-/-}17 and WT/AT^{-/-}42 are similarly proficient for NHEJ (as evidenced by zeocin resistance) WT/AT^{-/-}42 with high levels of DNA-PK can partially attenuate toxic NHEJ and is more Cpt resistant.

DNA-PK does not promote Cpt resistance in NHEJ deficient cells.

To assess whether or not increasing levels of DNA-PK activity might promote Cpt resistance in NHEJ deficient cells, Cpt sensitivity was assessed in XRCC4 deficient XR-1 cells that lack DNA-PKcs, ATM, or both. ATM and DNA-PKcs were each ablated in XR-1 cells; subsequently DNA-PKcs was ablated from one of the ATM deficient clones. Consistent with Balmus et al., ablation of ATM does not induce Cpt sensitivity in XRCC4 deficient XR-1 cells (Fig. 2) as it does in DNA-PKcs deficient V3 cells (Fig. 1). Moreover, loss of DNA-PKcs or both DNA-PKcs and ATM does not alter Cpt sensitivity in XR-1 cells. These data demonstrate that in ATM deficient cells, DNA-PK cannot promote further resistance in already resistant NHEJ deficient cells. DNA-PK can only promote Cpt survival in cells that have intact NHEJ. It follows (but is speculative) that phosphorylation of the ATM target [that we suggest is phosphorylated by DNA-PK in ATM deficient cells] inhibits toxic NHEJ.

The dominant negative-like effect (or “slow NHEJ”) that results from ABCDE blockade in cells exposed to camptothecin requires DNA-PKcs’s catalytic activity and phosphorylation of the PQR sites.

Our next series of experiments focused on interrogation of any relationship between the Cpt sensitivity that results from ATM ablation versus that induced by blocking phosphorylation of the ABCDE sites. We assessed whether the capacity for ABCDE-blocked DNA-PKcs to impair recovery from agents that induce replication associated single DNA ends also requires DNA-PK’s catalytic activity and phosphorylation of the PQR sites as is the case with agents that induce two-ended DSBs. As can be seen, cells expressing combination mutants that block both ABCDE and PQR sites, or mutants that block ABCDE and also ablate catalytic activity, function similar to cells lacking DNA-PKcs. We conclude that the increased sensitivity to Cpt by blocking ABCDE phosphorylation requires DNA-PK’s kinase activity as well as phosphorylation of the PQR sites. From our understanding of the ABCDE cellular phenotype [7, 8, 10, 14–18], we suggest that as is the case at two-ended DSBs, blocking ABCDE phosphorylation at single DNA ends that result from replication fork collapse, likely causes prolonged association of DNA-PKcs at the site of damage slowing DNA repair. Consistent with previous studies with ABCDE mutants, induction of H2AX phosphorylation is exaggerated and prolonged after exposure to Cpt (Fig. 3C)

Considerable progress has been made recently in advancing our understanding of DNA-PK’s structure [19, 25]. What is missing in all of these structures is information regarding the ~200 residue disordered region in DNA-PKcs where the ABCDE sites are located. We have previously proposed that blocking ABCDE phosphorylation (when PQR phosphorylation can proceed) results in a structural block of end processing [7, 10], such that Artemis is not activated and the DNA-PK complex cannot dissociate from DNA ends (illustrated in Fig. 3D). Since there is no structural information regarding the ABCDE disordered region, it is unclear whether this region actually directly impedes access to DNA ends (as depicted in the

left panel of cartoon, 3D), or instead promotes a conformation that impeded access to DNA ends). In any case, these data demonstrate that the “slow NHEJ” effect of ABCDE blockade at single DNA ends requires DNA-PK’s catalytic activity and phosphorylation of PQR sites analogous to its effect at two-ended DSBs.

Our previous studies concluded that both ABCDE and PQR phosphorylations were primarily autophosphorylations [9, 21]. These studies also demonstrated that ABCDE and PQR phosphorylation could occur in trans, although we suggested that both cis and trans autophosphorylations could occur. This question is still not completely resolved [26, 27]. In Figure 3C, cis autophosphorylation is suggested. Blocked topoisomerase I complexes induce collapsed replication forks and single double stranded ends; thus, implicit in these data is that autophosphorylation of ABCDE sites induced by Cpt, occurs at a single DNA-PK complex on a single DNA end.

Slow NHEJ rescues Cpt-sensitivity of ATM deficient cells.

To assess the impact of ABCDE blockade on toxic NHEJ associated with ATM deficiency, ATM was ablated (via Crispr/Cas9) in a V3 transfectant expressing the DNA-PKcs ABCDE>6Xala mutant (clone 7, ABCDE>A7). As can be seen, ablation of ATM in cells expressing the ABCDE>A7 mutant does not increase Cpt sensitivity (Fig. 4). Moreover, ATM deficient ABCDE>A7 clones are markedly more Cpt resistant than ATM deficient WT-52 clones, even though both ABCDE>A/AT–/– mutants tested express slightly less DNA-PKcs than the WT/AT–/–42 clone that is considerable more Cpt resistant than clones that express less DNA-PKcs (Fig. 1). We conclude that similar to ablating NHEJ, the “slow” NHEJ mediated by the ABCDE>A7 mutant rescues the Cpt hypersensitivity of ATM deficient cells, preventing toxic NHEJ and the associated genomic instability. In addition, since the ABCDE>A7 mutant retains full catalytic activity, the ABCDE mutant might facilitate Cpt resistance by phosphorylating the target responsible for blocking toxic NHEJ. In sum, these data suggest that although both blocking ABCDE phosphorylation and ablating ATM dramatically sensitize cells to Cpt, the defects in repair must be distinct.

Cpt-induced DNA-PKcs T2609 and S2056 phosphorylations are robust in ATM deficient 293T cells.

We have previously concluded [9] and more recently corroborated [21] that phosphorylation within both the ABCDE and PQR clusters is primarily autophosphorylation. Still, this conclusion is somewhat controversial with others concluding that ATM and ATR primarily phosphorylate the ABCDE cluster in response to DSBs and/or replication stress [28, 29]. Although one of the ABCDE phosphorylation sites was identified in one of two phospho-proteome experiments in the study from Balmus et al. [1], it was not robust and only modestly reduced by ATM inhibition [1]. Similarly, phospho-proteome studies from Elledge and colleagues concluded that IR-induced phosphorylation of ABCDE sites was not ATM dependent [30].

Because of the extremely high levels of DNA-PKcs in human cells, examination of DNA-PKcs phosphorylation is more straightforward than in rodent cells. Thus, we examined Cpt resistance and Cpt-induced DNA-PKcs phosphorylation in wild type and ATM deficient

293T cells characterized previously [5, 21]. As expected, and further corroborating the study of Balmus et al, ATM deficient 293T cells are markedly sensitive to Cpt (Fig. 5A).

Direct comparison of ABCDE versus PQR phosphorylation is not straightforward. Although phosphorylations within the ABCDE cluster are highly represented in phospho-proteome experiments [phospho-site.org lists 222 observations of phosphorylations across the ABCDE sites [31]], there are currently no commercially available reagents that have been validated to detect these phosphorylations. Although some previously available commercial reagents detect phosphorylation of T2609 and S2612, numerous other phosphoproteins with similar electrophoretic mobility are also detected with these antibodies in cells treated with DNA damaging agents [21]. In contrast, several excellent reagents are available to detect S2056 phosphorylation in the PQR cluster; however, peptides spanning the PQR sites are rarely observed by mass-spectrometry [phospho-site.org lists only 10 phosphorylations detected by high throughput methods across the PQR sites]. This is potentially explained by the large size of proteolytic fragments spanning the PQR sites. It is unlikely that this reflects any difference in actual phosphorylations, because detection of PQR phosphorylations using phospho-specific antibodies is reported more often than for any of the ABCDE sites [31]; of course this may also reflect the quality of available antibodies [21].

In our previous studies, (using a rabbit polyclonal anti-sera to phospho-T2609, generous gift of Dale Ramsden), only weak induction of T2609 phosphorylation (induced by either IR or DSB inducing drugs) is observed; detection of T2609 phosphorylation is strongly enhanced by treating cells with the phosphatase inhibitor okadaic acid [21]. We examined both S2056 and T2609 phosphorylation in wild type or ATM deficient 293T cells treated for 1 hour with 1 μ M Cpt, or 0.1nM calicheamicin (Fig. 5B). Consistent with our previous study, only weak induction of T2609 phosphorylation is observed in wild type 293T cells treated with calicheamicin. No significant T2609 phosphorylation is detected in wild type 293T cells treated with Cpt. In contrast, substantial T2609 phosphorylation is observed in ATM deficient 293T cells treated with either drug. This reagent also detects a damage-induced phospho-protein that has slower electrophoretic mobility than DNA-PKcs; phosphorylation of this protein is completely ablated in ATM deficient cells. Perhaps mistaking this protein for DNA-PKcs explains conclusions in previous studies, that 2609 phosphorylation is ATM dependent. S2056 phosphorylation is minimally detected in wild type 293T cells treated with Cpt, whereas S2056 phosphorylation is clearly apparent in ATM deficient 293T cells. These data demonstrate that Cpt-induced phosphorylation of T2609 does not require ATM. Moreover, Cpt-induced phosphorylation of both S2056 and T2609 is increased in ATM deficient cells, perhaps a reflection of “toxic NHEJ” in ATM deficient cells. Since we show that ABCDE phosphorylation is requisite for toxic NHEJ in ATM $-/-$ cells, it seems unlikely that ATM phosphorylation of DNA-PK’s ABCDE sites at Cpt-induced collapsed replication forks would impair NHEJ in ATM proficient cells. Moreover, if ATM phosphorylation of ABCDE is important to prevent toxic NHEJ, then ABCDE blockade should phenocopy ATM deficiency. It clearly does not.

ATM phosphorylation of DNA-PKcs at S3205 facilitates camptothecin resistance.

In the study of Balmus et al., another DNA-PKcs phosphorylation (S3205) was reported that was consistently identified, was robust, and was strongly reduced in the presence of an ATM inhibitor. This is consistent with our previous results showing that IR-induced phosphorylation of S3205 is ATM dependent [10]. Although we have previously demonstrated that blocking S3205 does not dramatically impact NHEJ in response to agents that induce DSBs, the impact of blocking S3205 in cells exposed to agents that induce replication associated DNA damage (like camptothecin) was not studied.

In all DNA damage induced kill curve assays presented previously, the S3205A and S3205D mutants behave similarly, suggesting that the S3205D mutant does not actually mimic phosphorylation [17]. As with previous studies utilizing IR or zeocin, cells expressing the S3205A and S3205D mutants are similarly resistant to cells expressing wild type DNA-PKcs to the DSB-inducing drug calicheamicin (Fig. 6B). As expected, cells lacking DNA-PKcs or expressing the ABCDE mutant are markedly sensitive to calicheamicin (Fig. 6B). In contrast, both S3205 mutants are more sensitive to camptothecin than cells expressing either wild type DNA-PKcs or no DNA-PKcs (Fig. 6C), but as with agents that induce DSBs, the phospho-blocking and phospho-mimicking mutants behave identically, suggesting that the phospho-mimic mutant does not actually mimic phosphorylation. In sum, these data suggest that phosphorylation of S3205 (that is ATM dependent) facilitates repair of Cpt-induced DNA damage.

We next ablated ATM in the cell line expressing the S3205>A mutant; two clones were tested. As can be seen, ATM deficient cells expressing S3205>A are somewhat more sensitive to Cpt than the WT/AT^{-/-}42 clone, even though one of the two clones (3205>A/AT^{-/-}16) expresses more DNA-PK than WT/AT^{-/-}42 (Fig. 6D and 6E). We have shown previously that DNA-PK can autophosphorylate S3205 *in vitro* [17, 32]; we suggest that in ATM deficient cells, “back-up” DNA-PKcs autophosphorylation of S3205 facilitates cellular resistance to Cpt.

S3205 phosphorylation is required for the “slow NHEJ” phenotype at single DNA ends, but not two-ended DSBs.

We next considered whether phosphorylation of S3205 might commit cells to the ATM dependent pathway that promotes homology directed repair. Although blockade of the ABCDE sites renders cells resistant to toxic NHEJ associated with ATM deficiency, these cells are still quite sensitive to Cpt. We have previously shown that blocking S3205 phosphorylation in ABCDE blocked cells does not alter resistance to DSB-inducing agents like IR and zeocin [10]. We next considered whether S3205 phosphorylation of ABCDE blocked DNA-PK might commit Cpt treated cells to repair by ATM dependent HDR. We assessed Cpt and calicheamicin sensitivity of cells expressing ABCDE>A7 versus ABCDE +3205>A (clone 7, ABCDE>A+S3205>A7) (Fig. 7). Consistent with our previous studies [10], ablation of S3205 in the ABCDE mutant, does not impact the slow NHEJ and hypersensitivity to the DSB-inducing agent calicheamicin. In contrast, ablation of S3205 in the ABCDE mutant (ABCDE>A+S3205>A7) completely reverses cellular sensitivity to Cpt in a similar manner as blocking ABCDE sites reverses Cpt sensitivity in ATM deficient

cells. We infer from these data that the increased occupancy of ABCDE blocked DNA-PKcs at single DNA ends associated with collapsed replication forks, requires phosphorylation of S3205 in DNA-PKcs. These data suggest a model whereby ATM phosphorylation of DNA-PKcs at S3205 facilitates repair of Cpt-induced DNA damage.

Discussion

Here we expand upon the concept of toxic NHEJ that results at collapsed replication forks in ATM deficient cells [1]. First, our data suggest that DNA-PK can partially compensate for ATM loss and that the ability of DNA-PK to prevent toxic NHEJ in ATM deficient cells is directly correlated to DNA-PKcs expression levels. Although speculative, these data suggest that DNA-PK phosphorylates an ATM target that is required to prevent toxic NHEJ.

Second, we demonstrate that “toxic NHEJ” and “slow NHEJ” that result from blocking ABCDE phosphorylation occur by distinct mechanisms; moreover, “slow NHEJ” rescues cells from toxic NHEJ similar to ablation of NHEJ. These somewhat disparate results could be explained as follows: whereas phosphorylation of ABCDE is required to facilitate repair at collapsed forks (perhaps by promoting Mre11-mediated removal of DNA-PK [4]), blocking ABCDE phosphorylation protects ATM deficient cells from dysregulated NHEJ joining of single DNA ends at collapsed forks, “toxic NHEJ”.

Finally, we show that S3205 phosphorylation (a site documented numerous times to be phosphorylated *in vivo* [31], and shown previously to be phosphorylated by ATM [1, 10]) promotes survival after Cpt-induced damage. A potential model would be that S3205 in some way limits mobility of the single DNA end, so that synapsis of two DNA ends from different collapsed forks (a presumed requirement for “toxic NHEJ”), does not occur. Moreover, S3205 phosphorylation is required for the “slow NHEJ” phenotype in ABCDE>A7 cells treated with agents that induce single DNA ends, but not two-ended DSBs. Finally, blocking S3205 phosphorylation enhances “toxic NHEJ” in ATM deficient cells, providing evidence that this site might be a “back up” target phosphorylated by DNA-PK in ATM deficient cells. All together, these data suggest a working model whereby ATM phosphorylation of S3205 at collapsed forks, blocks toxic NHEJ, perhaps stabilizing DNA-PK at the single DNA end.

In sum, these conclusions and emerging new data from Paull and colleagues [4] suggest our working model (Fig. 8) that DNA-PK and ATM cooperate to facilitate repair at collapsed replication forks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ATM deficient cells are remarkably sensitive to topoisomerase poisons like camptothecin (Cpt); this hypersensitivity results from unrestrained end-joining of DNA ends at collapsed replication forks that is mediated by the non-homologous end joining [NHEJ] pathway and results in the induction of copious numbers of genomic alterations, termed “toxic NHEJ”. This manuscript expands our understanding of how ATM prevents “toxic NHEJ” providing evidence that DNA-PK and ATM cooperate to facilitate repair at collapsed replication forks.

Figure 1. DNA-PKcs contributes to toxic NHEJ; but increasing levels of DNA-PK promote Cpt resistance in ATM deficient NHEJ proficient cells in a titratable manner.

A. Cartoon depicting ATM's inhibition (by an unknown mechanism) of toxic NHEJ at Cpt-induced collapsed replication forks. **B.** Immunoblot analyses of 50ug whole cell extracts from V3 clonal transfectants expressing wild type human DNA-PKcs (WT-52) or no DNA-PKcs (Vect-5) from which ATM was ablated via Crispr/Cas9 or not. **C.** V3 clones were plated at cloning densities into complete medium with increasing doses of Zeocin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean. **D.** V3 clones were plated into 24 well plates in complete medium with increasing doses of camptothecin. Cells were stained with MTT after seven days, and percent survival was calculated. Error bars represent the standard error of the mean.

Figure 2. DNA-PK does not promote Cpt resistance in NHEJ deficient cells.

A. Immunoblot analyses of 50ug whole cell extracts from XR1 cells or XR1 cells from which ATM was ablated (ATM^{-/-5}, ATM^{-/-22}), or DNA-PKcs was ablated (DNA-PKcs^{-/-5}, DNA-PKcs^{-/-15}), or both DNA-PKcs and ATM were ablated (ATM/DNA-PKcs^{-/-26}). Filter was probed first with anti-ATM antibody (top panel) and then with anti-DNA-PKcs antibody (bottom panel). A non-specific band (*) serves as a loading control. **B.** XR-1 clones were plated at cloning densities into complete medium with increasing doses of camptothecin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean.

Figure 3. The dominant negative-like effect (or “slow NHEJ”) that results from ABCDE blockade in cells exposed to camptothecin requires DNA-PK’s catalytic activity and phosphorylation of the PQR sites.

A. Immunoblot analyses of 50ug whole cell extracts from V3 clonal transfectants expressing wild type human DNA-PKcs (WT-52), or DNA-PKcs mutants ABCDE>A (ABCDE>A-7), ABCDE+PQR+S3205A>Ala (ABCDE+PQR+3205>A-6), ABCDE>A+kinase dead (ABCDE>A/K>R-5), or no DNA-PKcs (Vect-5). **B.** V3 clones were plated at cloning densities into complete medium with increasing camptothecin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean. **C.** Immunoblot analyses of whole cell lysates of WT-52 or ABCDE>A7 cells either untreated or treated with 1 μ M Cpt for 1, 4, or 24 hours as indicated. **D.** Cartoons depicting DNA end protection by DNA-PK when ABCDE sites are blocked, and PQR sites are phosphorylated (left) or DNA end accessibility promoted by DNA-PK with phosphorylated ABCDE sites.

Figure 4. Slow NHEJ rescues Cpt-sensitivity of ATM deficient cells.

A. Immunoblot analyses of 50ug whole cell extracts from V3 clonal transfectants expressing wild type human DNA-PKcs (WT-52), or DNA-PKcs wild type from which ATM was ablated (WT/AT-/-42), or DNA-PKcs mutant ABCDE>A (ABCDE>A-7), or ABCDE>A mutant from which ATM was ablated (ABCDE>A/AT-/-49, ABCDE>A/AT-/-31). **B.** V3 clones were plated into 24 well plates in complete medium with increasing doses of camptothecin. Cells were stained with MTT after seven days, and percent survival was calculated. Error bars represent the standard error of the mean.

Figure 5. Cpt-induced DNA-PKcs T2609 and S2056 phosphorylations are robust in ATM deficient 293T cells.

A. 293T cells were plated into 24 well plates in complete medium with increasing doses of camptothecin. Cells were stained with MTT after seven days, and percent survival was calculated. Error bars represent the standard error of the mean. **B.** Immunoblot analyses of 50ug whole cell extracts from wild type ATM deficient cell treated with either 1uM camptothecin or 0.1 nM calicheamicin for 1 hour.

Figure 6. ATM phosphorylation of DNA-PKcs at S3205 facilitates camptothecin resistance.

A. Immunoblot analyses of 50ug whole cell extracts from V3 clonal transfectants expressing wild type human DNA-PKcs, DNA-PKcs mutants ABCDE>A, S3205>Ala, S3205>Asp, or no DNA-PKcs (vector). **B.** V3 clones were plated at cloning densities into complete medium with increasing doses of calicheamicin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean. **C.** V3 clones were plated at cloning densities into complete medium with increasing doses of camptothecin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean. **D.** Immunoblot analyses of 50ug whole cell extracts from V3 clonal transfectants expressing wild type human DNA-PKcs (WT-52), or DNA-PKcs wild type from which ATM was ablated (WT/AT-/-42), or DNA-PKcs mutant S3205A (S3205>A8), or S3205A mutant from which ATM was ablated (S3205>A/AT-/-16 or S3205>A/AT-/-3). **E.** V3 clones were plated at cloning densities into complete medium with increasing doses of camptothecin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean.

Figure 7. S3205 phosphorylation is required for the “slow NHEJ” phenotype at single DNA ends, but not two-ended DSBs.

A. Immunoblot analyses of 50ug whole cell extracts from V3 clonal transfectants expressing wild type human DNA-PKcs, DNA-PKcs mutants ABCDE>A, S3205>Ala, ABCDE>A +S3205>Ala, or no DNA-PKcs (vector). **B.** V3 clones were plated at cloning densities into complete medium with increasing doses of calicheamicin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the mean. **C.** V3 clones were plated at cloning densities into complete medium with increasing doses of camptothecin. Colonies were stained after seven days, and percent survival was calculated. Error bars represent the standard error of the means.

Figure 8.
DNA-PK and ATM cooperate to facilitate repair at collapsed replication forks.