Essential role for DNA-PKcs in DNA double strand break repair and apoptosis in ATM deficient lymphocytes

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SUMMARY

The DNA double strand break (DSB) repair protein DNA-PKcs and the signal transducer ATM are both activated by DNA breaks and phosphorylate similar substrates *in vitro*, yet appear to have distinct functions *in vivo*. Here we show that ATM and DNA-PKcs have overlapping functions in lymphocytes. Ablation of both kinase activities in cells undergoing immunoglobulin class switch recombination leads to a compound defect in switching, and a synergistic increase in chromosomal fragmentation, DNA insertions and translocations due to aberrant processing of DSBs. These abnormalities are attributed to a compound deficiency in phosphorylation of key proteins required for DNA repair, class switching and cell death. Notably, both kinases are required for normal levels of p53 phosphorylation in B and T cells and p53 dependent apoptosis. Our experiments reveal a DNA-PKcs-dependent pathway that regulates DNA repair and activation of p53 in the absence of ATM.

INTRODUCTION

ATM and ATR are signal transducing phosphatidyl inositol 3-kinase-like kinases (PIKKs) that are central to the DSB response (Rouse and Jackson, 2002). At least 700 proteins are phosphorylated on ATM/ATR consensus sites in response to irradiation (IR) (Matsuoka et al., 2007). A third member of the PIKK family, DNA-PKcs, is itself a target of ATM/ATR (Chen et al., 2007; Matsuoka et al., 2007), and is thought to play a more restricted role in DSB repair (Kurimasa et al., 1999). DNA-PKcs is essential for nonhomologous end-joining (NHEJ), a pathway that promotes cell survival in response to irradiation (Smith and Jackson, 1999) and repairs programmed DSBs created during V(D)J recombination and class switch

recombination (CSR) in lymphocytes (Dudley et al., 2005; Jankovic et al., 2007; Smith and Jackson, 1999). Although PIKKs recognize similar target consensus sites *in vitro*, the physiological substrate(s) of DNA-PKcs that facilitates NHEJ remain unknown.

ATM and DNA-PKcs are both recruited to and activated by DSBs. ATM is targeted to DSBs through an interaction with the DSB sensor Mre11/Rad50/Nbs1 complex (Lee and Paull, 2005), whereas DNA-PKcs recruitment is dependent on prior loading of the Ku70/80 heterodimer on DNA ends(Dvir et al., 1992; Gottlieb and Jackson, 1993). In contrast, the molecular species recognized by ATR is single stranded DNA (ssDNA) coated by replication protein A (RPA), a ssDNA binding protein (Zou and Elledge, 2003). Maximal ATR activation is dependent on ATM and on Mre11-dependent nucleolytic processing of DSBs into ssDNA (Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006; Yoo et al., 2007).

While most DSB signal transduction pathways are thought to be governed cooperatively by ATM and ATR, recent studies raise the possibility of cross-talk between DNA-PKcs and ATM in DNA damage signaling. For example, a DNA-PKcs dependent component to the G2/M checkpoint has been reported in epithelial cells (Arlander et al., 2008; Liu et al., 2008), and H2AX, RPA and DNA-PKcs phosphorylation is co-dependent on ATM and DNA-PKcs in irradiated fibroblasts and tumor cell lines (Chen et al., 2007; Stiff et al., 2004; Wang et al., 2001). However, combined inactivation of ATM and DNA-PKcs does not yield an additive DSB joining defect in fibroblasts (Riballo et al., 2004), nor increased radiation sensitivity in cancer cell lines (Cowell et al., 2005) compared to loss of either kinase alone.

Several lines of evidence indicate that ATM and DNA-PKcs play distinct roles in the DSB response *in vivo*. First, compared with the very broad spectrum of ATM substrates, the targets of DNA-PKcs *in vivo* remain undefined. Second, the phenotypes associated with ATM and DNA-PKcs deficiencies are distinct. ATM knockout mice are sterile due to a block in meiosis, lymphopenic and predisposed to lymphomas, whereas DNA-PKcs knockout mice are fertile but exhibit severe combined immunodeficiency (SCID) without increased incidence of tumorigenesis. Third, combined deficiency in DNA-PKcs and ATM results in synthetic lethality early in embryogenesis (Gurley and Kemp, 2001; Sekiguchi et al., 2001). Finally, whereas DNA damage induced cell cycle and apoptotic checkpoints are severely compromised in cells lacking ATM (Shiloh, 2003), DNA-PKcs is dispensable for DSB signaling, p53 activation and cell cycle arrest (Burma et al., 1999; Jhappan et al., 2000; Jimenez et al., 1999; Rathmell et al., 1997). Thus, the majority of the evidence indicates that although ATM and DNA-PKcs are both activated by IR induced DSBs they cannot substitute for each other in the DNA damage response *in vivo*, potentially because of distinct physiological targets.

In addition to IR induced DSBs, physiological DSBs are processed during class switch recombination in mature B cells in a reaction that alters the effector properties of antibodies (Barreto et al., 2005; Chaudhuri et al., 2007; Di Noia and Neuberger, 2007; Honjo et al., 2005; Li et al., 2007; Stavnezer et al., 2008). CSR is initiated by activation-induced cytidine deaminase (AID) (Muramatsu et al., 2000; Muramatsu et al., 1999; Revy et al., 2000), an enzyme that deaminates cytosine residues in ssDNA, leading to DSBs in switch regions which lie upstream of each of the constant region genes (Chaudhuri et al., 2003; Dickerson et al., 2003; Petersen-Mahrt et al., 2002; Pham et al., 2003; Ramiro et al., 2003). AID dependent DNA breaks trigger a DNA damage response, evidenced by the recruitment of DNA damage response proteins Nbs1, MDC1, ATM, γ-H2AX, and 53BP1 to IgH chromatin in the G1 phase of the cell cycle (Jankovic et al., 2007). Each of these factors is required for normal resolution of the CSR reaction (Difilippantonio et al., 2008; Franco et al., 2006; Jankovic et al., 2007; Ramiro et al., 2006; Reina-San-Martin et al., 2003). ATM and 53BP1 have been proposed to play a role in switch region synapsis during CSR (Lumsden 2004; Reina-San-Martin 2004;

Manis, 2004 #25; Ward, 2004 #27; Reina-San-Martin, 2007 #78}, while DNA-PKcs is implicated in joining of the subset of AID induced DSBs (Franco et al., 2008).

Here we report that DNA-PKcs and ATM coordinately control programmed DSB repair during class switch recombination and cell death in lymphocytes suggesting a broader role for DNA-PKcs beyond end-joining in the maintenance of genomic stability.

RESULTS

DNA-PKcs catalytic activity is essential for CSR in the absence of ATM

To investigate the relationship between DNA-PKcs and ATM we used a well characterized inhibitor NU7026 (PKi) to interfere with DNA-PKcs kinase activity in B cells (Leahy et al., 2004; Veuger et al., 2003). In agreement with studies in fibroblast cell lines, we found compromised IR induced H2AX phosphorylation in ATM$^{-/-}$ but not in wild type B cells that were pre-incubated with PKi (Figure S1)(Riballo et al., 2004; Stiff et al., 2004). Potential off-target effects of PKi on other PIKKs were not evident based on the robust IR-and replication stress-induced substrate phosphorylations in PKi treated cells (Figures S2 and S3).

ATM$^{-/-}$ B cells show decreased CSR but normal levels of cell division in response to LPS and IL-4 (Lumsden et al., 2004; Reina-San-Martin et al., 2004) (Figure 1A). B cells fail to develop in DNA-PKcs$^{-/-}$ due to absence of V(D)J recombination, but can be reconstituted by introduction of pre-rearranged Ig heavy and light chain transgenes (DNA-PKcs$^{-/-}$HL). In our experiments, DNA-PKcs$^{-/-}$HL B cells showed defects in CSR to IgG1 and IgG3 ranging from 30-70% of controls (Cook et al., 2003; Franco et al., 2008; Manis et al., 2002) (Figure 1A; Figure S4).

To examine the role of DNA-PKcs in CSR, B cells were labeled with CFSE [5-(and 6-) carboxyfluorescein diacetate succinyl ester] to track cell division by dye dilution and stimulated with LPS and IL-4 to induce switching to IgG1 in the presence or absence of PKi. We found a small increase in CSR to IgG1 in PKi treated wild type and DNA-PKcs$^{-/-}$HL B cells but no change in cell division (Figure 1B; Figure S5). Thus, while loss of the DNA-PKcs protein impairs CSR, inhibition of DNA-PKcs activity does not.

To examine the possibility that DNA-PKcs activity might be redundant with ATM during CSR we examined switching to IgG1 and IgG3 by ATM$^{-/-}$ B cells in the presence or absence of PKi. In contrast to wild type B cells, reducing DNA-PKcs activity in ATM$^{-/-}$ B cells resulted in a decrease in CSR while cell division remained intact (Figure 1B; Figure S5). This decrease in CSR was not due to altered AID protein levels or switch region accessibility as measured by germline transcription of switch regions (Figure S5). Thus, ATM and DNA-PKcs do not appear to affect events upstream of AID-mediated switch lesion formation. We conclude that ATM and DNA-PKcs have overlapping functions during CSR.

Loss of ATM and DNA-PKcs activity results in DNA insertions into CSR junctions

To determine whether loss of both PIKK activities impacts on DNA joining at immunoglobulin switch (S) regions we cloned and sequenced Sμ-Sγ1 junctions from IgG1 positive B cells that have completed CSR (Figure 2A). Consistent with previous analyses, we found no statistically significant alterations in the switch junctions in ATM deficient cells (Reina-San-Martin et al., 2004). However, loss of ATM resulted in a small increase in junctions showing three or more nucleotide insertions (Figure 2A). Such junctions are rare in wild type cells and the addition of PKi to wild type cells resulted in a small increase in these events (Figure 2A). Strikingly, addition of PKi to ATM$^{-/-}$ B cells increased the frequency of insertions to 32% of the total CSR junctions analyzed (Figure 2A). Many of these insertions were large (~100-200bp), and came from non-contiguous regions of switch DNA, consistent with the idea that AID introduces

multiple lesions in switch regions (Figure 2B)(Dudley et al., 2005; Reina-San-Martin et al., 2003). We conclude that DNA-PKcs and ATM have a redundant function in switch break repair, and are required to prevent intra-molecular insertions at the switch junctions. A similar function has been ascribed to 53BP1 (Difilippantonio et al., 2008; Manis et al., 2004; Reina-San-Martin et al., 2007).

Synergistic increase in genomic instability

DSBs are essential intermediates during antigen receptor gene diversification reactions, but they are also substrates for chromosomal translocations. As a result of their end-joining defects, DNA-PKcs$^{-/-}$ and ATM$^{-/-}$ B cells stimulated to undergo CSR accumulate IgH -specific chromosome breaks and general instability outside of the IgH locus (Callen et al., 2007; Franco et al., 2006; Franco et al., 2008; Ramiro et al., 2006). Similar defects are also found in wild type B cells treated with the small molecule inhibitor of ATM (KU55933) (Callen et al., 2007). To determine whether ATM and DNA-PKcs are redundant in maintaining genomic integrity during CSR we compared the level of genomic instability in wild type or ATM$^{-/-}$ B cells cultured in the presence or absence of PKi. *IgH* associated breaks, translocations, and general instability outside of the *IgH* locus were monitored by fluorescent in situ hybridization (FISH) using chromosome 12, telomere, and IgHCα-specific probes (Callen et al., 2007; Franco et al., 2006) (Figure 3A). In three independent experiments, wild type B cells showed low levels of general chromosome instability, and little or no *IgH* instability in the presence or absence of PKi (Figure 3A). As previously reported, 10-20% of ATM$^{-/-}$ B cells harbored *IgH* specific breaks, 0.5-3.5% carried *IgH* translocations and 20-30% had non-*IgH* associated instability (Figure 3A) (Callen et al., 2007). In marked contrast to wild type, inhibition of DNA-PKcs in ATM$^{-/-}$ B cells led to a synergistic increase in *IgH* breaks, translocations, and general genomic instability (Figure 3A). We conclude that DNA-PKcs and ATM are redundant for the switch reaction and for maintaining general and *IgH* chromosome stability in B cells undergoing CSR.

Increase in *c-myc/IgH* translocations

While both ATM and DNA-PKcs have been implicated in the repair of a subset of AID-induced DSBs (Franco et al., 2006; Franco et al., 2008; Ramiro et al., 2006; Reina-San-Martin et al., 2004), ATM and p53 have an additional apoptotic function: protecting against CSR-associated tumor promoting translocations between *c-myc* and *IgH* (Ramiro et al., 2006). In contrast, DNA-PKcs$^{-/-}$ B cells have an intact p53 dependent apoptotic checkpoint, evidenced by the finding that LPS stimulated DNA-PKcs$^{-/-}$ B cells that suffer DNA damage are eliminated by p53 (Franco et al., 2008).

To determine whether ATM and DNA-PKcs also serve a redundant function in protecting against *c-myc/IgH* translocation, we assayed for the frequency of these events in ATM$^{-/-}$ B cells treated with PKi (Ramiro et al., 2006; Ramiro et al., 2004) (Figure 3B). Consistent with the effects on CSR, we found a significant increase in the frequency of *c-myc/IgH* translocations in ATM$^{-/-}$ B cells treated with PKi compared to ATM$^{-/-}$ B cells alone (Figure 3B). Therefore, DNA-PKcs kinase activity does indeed protect against *c-myc/IgH* translocation in the absence of ATM.

Impaired phosphorylation of DNA repair and checkpoint proteins

Combined inhibition of ATM and DNA-PKcs resembles deficiency in 53BP1(Manis et al., 2004; Ramiro et al., 2006; Reina-San-Martin et al., 2007; Ward et al., 2004) in that it results in dramatically decreased CSR, switch region insertions, and *IgH* instability (Figures 1-3). To determine whether combined PIKK inhibition alters 53BP1 responses we measured its phosphorylation and focus formation in response to irradiation (Figures 4A and 4B). Inhibition of DNA-PKcs or ATM deficiency resulted in decreased 53BP1 Ser25 phosphorylation, and

the combination led to the complete absence of phosphorylation (Figure 4A). In addition, PKi abrogated 53BP1 focus formation in ATM^{-/-} but not in wild type B cells exposed to IR (Figure 4B). Thus, ATM and DNA-PKcs phosphorylate 53BP1 and regulate 53BP1 focus formation in a redundant manner.

To determine whether ATM and DNA-PKcs collaborate in phosphorylating additional downstream substrates, we examined the effects of combined loss of the two PIKKs on the classical ATM substrates Kap-1, Chk2, Chk1, p53 and SMC1 in irradiated B cells and thymocytes. We found that IR induced phosphorylation of Kap-1, SMC1, Chk2, p53 and Chk1 was further compromised in ATM^{-/-} B cells or thymocytes treated with PKi (Figures 4C-D; Figure S6). These data suggest that ATM and DNA-PKcs are generally redundant for substrate phosphorylation in mouse lymphocytes.

To further confirm these findings, we targeted DNA-PKcs in primary B cells with short hairpin RNAs (shRNAs). Western blot analysis revealed reduction of DNA-PKcs after infection with two independent shRNAs, PKsh61 and PKsh62 (Figure 4E). Consistent with the results with PKi, downregulation of DNA-PKcs in ATM deficient cells led to further reduction in IR induced phosphorylation of Kap-1, p53 and 53BP1 (Figure 4E). Finally, we used a genetic approach to produce B cells deficient in ATM and DNA-PKcs. DNA-PKcs^{-/-}HL and ATM conditional knock out mice (Zha et al., 2008)(Zha et al, in preparation) were bred to generate DNA-PKcs^{-/-}HL/ATM[c/c] B cells. To delete ATM activity, mutant B cells were infected with a virus expressing cre recombinase or vector control expressing GFP as a marker. GFP^{+} cells were sorted, and efficient deletion of ATM was confirmed by PCR (Figure S7). The sorted cells were irradiated and analyzed by Western blotting. Whereas ATM or DNA-PKcs deficient cells are capable of phosphorylating Kap1-1 and H2AX in response to IR (Figure 4F), deletion of ATM in DNA-PKcs knockout B cells led to a near complete ablation of in Kap-1 and H2AX phosphorylation (Figure 4F). Thus, combined genetic depletion of ATM and DNA-PKcs confirms that the two kinases are redundant in lymphocytes.

To determine whether the functional overlap in lymphocytes extends to other cell types we analyzed Kap-1 phosphorylation in mouse embryo fibroblasts (MEFs). DNA-PKcs activity was depleted from ATM^{-/-} immortalized MEFs either by treatment with PKi (Figure 5A), or by PKcs shRNA (Figure 5B), or by adenoviral CRE infection of ATM[c/c]DNA-PKcs^{-/-} primary MEFs (Figure 5C). In all cases, combined deficiency in ATM and DNA-PKcs led to a reduction in Kap-1 phosphorylation relative to that of ATM deficiency alone (Figures 5A-C). However, in contrast to lymphocytes, DNA-PKcs did not affect the activation of p53 in primary ATM^{-/-} MEFs treated with 20 Gy irradiation (Figure 5C). Consistent with this result, it has been reported that ATR cooperates with ATM in the IR induced phosphorylation of p53 in primary MEFs (Brown and Baltimore, 2003). Differences in the degree of redundancy between ATM, DNA-PKcs and ATR may reflect differences in the proportion of cycling cells (see discussion) or differences in the levels of DNA-PKcs in different cell types (Figure 5D).

Collaboration between ATM and DNA-PKcs in p53 dependent apoptosis

DNA-PKcs is not known to phosphorylate p53 *in vivo* (Jimenez et al., 1999); on the other hand, it does contribute to IR induced p53 activation in lymphocytes lacking ATM (Figure 4D; Figure S6). Therefore we speculated that DNA-PKcs might be an important inducer of apoptosis in irradiated lymphocytes lacking ATM. To test this hypothesis, we pre-treated ATM^{-/-} and wild type thymocytes with PKi, and measured cellular viability by flow cytometry 24 hours after irradiation (Figure 6A). We found that PKi protected irradiated ATM^{-/-} cells to levels approaching p53^{-/-}, whereas viability of wild type thymocytes was unaffected by PKi (Figure 6A; Figure S8). We conclude that DNA-PKcs is essential for triggering p53 dependent apoptosis in response to irradiation in ATM^{-/-} thymocytes.

Irradiation induced thymocyte apoptosis is regulated in part through p53 dependent induction of the pro-apoptotic genes NOXA, PUMA, and BAX. To gain insight into the basis for the apoptotic defect in ATM$^{-/-}$ thymocytes we examined the expression of these genes by real time quantitative RT-PCR four hours after treatment with 5 Gy of irradiation. The irradiation induced increase in messenger RNA for these genes was not significantly different between ATM$^{-/-}$ and wild type thymocytes (Figure 6B). However, pre-treatment of ATM$^{-/-}$ thymocytes with PKi completely abrogated the induction of pro-apoptotic genes as well as the induction of the p21 cell cycle inhibitor, all of which accumulate in a p53-dependent manner (Figure 6B). Thus, DNA-PKcs is required for ATM-independent transcriptional activation of p53 dependent genes in lymphocytes.

DISCUSSION

The ATM and ATR signaling network regulates replication fork stability, transcription, checkpoint signaling, and DNA repair. In contrast, DNA-PKcs is thought to have a more restricted role in the re-ligation of broken ends by NHEJ (Smith and Jackson, 1999). Our studies reveal a broader function for the DNA-PKcs dependent pathway in the DNA damage response in lymphocytes.

Non-catalytic role of DNA-PKcs in CSR

DNA-PKcs catalytic activity is reported to be essential for V(D)J recombination (Kurimasa et al., 1999) and SCID thymocytes lacking DNA-PKcs activity exhibit an increase in p53 activation and apoptotic cell death (Guidos et al., 1996). The role of DNA-PKcs in CSR is the subject of debate with some investigators reporting near normal switching in SCID mice (Bosma et al., 2002) and in DNA-PKcs$^{-/-}$ mice (Kiefer et al., 2007), while others have demonstrated variable levels of impairment in CSR (Cook et al., 2003; Manis et al., 2002) with the most profound defects found in null mutant mice (Manis et al., 2002). Our experiments confirm the existence of partial CSR defects to both IgG1 and IgG3 in mice lacking DNA-PKcs protein. In contrast, CSR or end-joining of *IgH* switch region breaks was not impaired in wild type cells depleted of DNA-PKcs catalytic activity. Our results suggest that in ATM sufficient cells DNA-PKcs has functions other than catalysis in promoting CSR. For example, DNA-PKcs may play a structural role acting as a scaffold to align, synapse or protect CSR breaks (DeFazio et al., 2002).

Impaired CSR in the absence of DNA-PKcs and ATM resembles 53BP1 deficiency

While DNA-PKcs enzymatic activity is dispensable for end-joining during CSR in normal cells, similar reduction of DNA-PKcs activity in ATM$^{-/-}$ B cells results in decreased class switching, accumulation of intra-molecular insertions at switch junctions, and a synergistic increase in *IgH* and general chromosomal instability. Our study suggests that these observations can be explained by a previously unappreciated compound defect in phosphorylation of DNA repair and damage signaling proteins in doubly deficient lymphocytes as discussed below.

ATM plays a central role in DSB repair by phosphorylating numerous substrates including focus forming factors Nbs1, MDC1, H2AX, and 53BP1 and NHEJ proteins that also participate in CSR break repair (Jankovic et al., 2007). Among these factors, the most profound defect in CSR is found in 53BP1 deficient B cells, which show a nearly complete block in the reaction (Manis et al., 2004; Reina-San-Martin et al., 2007; Ward et al., 2004). 53BP1$^{-/-}$ cells undergo a perturbed form of the CSR reaction evidenced by an increase in short range intra-switch region joints and a concomitant decrease in long range junctions that contain insertions of non-contiguous switch region DNA (Reina-San-Martin et al., 2007). Similar but less pronounced defects in CSR are also found in ATM, H2AX, Nbs1 and DNA-PKcs deficient B cells.

However, in contrast to 53BP1 deficiency, the CSR junctions in these mice are nearly normal (Celeste et al., 2002; Kracker et al., 2005; Lumsden et al., 2004; Manis et al., 2002; Reina-San-Martin et al., 2004; Reina-San-Martin et al., 2003; Reina-San-Martin et al., 2005). Our finding that inhibition of DNA-PKcs activity in ATM$^{-/-}$ abrogates 53BP1 phosphorylation and focus formation, and results in CSR junctions that resemble those found in 53BP1$^{-/-}$ B cells, suggests that the two kinases can substitute for each other in activating key DNA damage response factors required for the repair of DSBs. This idea is further supported by cooperative phosphorylation of Kap-1 and SMC1, both of which have been implicated in chromosomal repair and cell survival after DNA damage (Kitagawa et al., 2004; Ziv et al., 2006).

Synergistic increase in genomic instability

In addition to decreased CSR, loss of ATM leads to genomic instability and an increase in AID mediated *c-myc/IgH* translocations (Callen et al., 2007; Ramiro et al., 2006). These aberrations are further increased by loss of DNA-PKcs catalytic activity. B cells containing chromosomal breaks or oncogenic translocations are normally eliminated via a p53 dependent pathway, which is thought to be activated in part by ATM (Ramiro et al., 2006). Our experiments show that ATM and DNA-PKcs are redundant with respect to activation of p53 and the Chk2 pathway. The combined deficiency of the two PIKKs results in a severe defect in DNA repair and activation of apoptosis, which we propose contributes to the synergistic increase in chromosomal aberrations in B cells lacking ATM and DNA-PKcs.

Upon DNA damage, the p53 tumor suppressor undergoes ATM- and Chk2-dependent phosphorylation, leading to increased transcription of target genes that promote apoptosis. It has been suggested that ATR may contribute to p53 mediated apoptosis because there is a partial defect in p53 stabilization in irradiated ATM and Chk2 deficient mice (Hirao et al., 2002; Takai et al., 2002); furthermore, there is a requirement for phosphorylation of SQ/TQ sites in Chk2 to induce ATM independent p53 activation (Hirao et al., 2002); and finally p53 activation in primary MEFs is co-dependent on ATM and ATR (Brown and Baltimore, 2003). In contrast, our results strongly suggest that ATM and DNA-PKcs collaborate to promote p53 activation in irradiated thymocytes and B cells undergoing CSR. This is consistent with the findings that ATR signaling is subject to cell cycle control, occurring primarily in S/G2 (Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006; Yoo et al., 2007), whereas the vast majority of irradiation induced breaks in thymocytes, and physiological AID dependent breaks in B cells, are found in the G1 phase of the cell cycle (Petersen et al., 2001; Schrader et al., 2007). Moreover, we have found that even the residual IR induced ATR activation in thymocytes is co-dependent on ATM and DNA-PKcs, as evidenced by the abrogation of ATR dependent Chk1 phosphorylation in cells with compromised ATM and DNA-PKcs function (Figure S6B). A larger population of S phase cells in MEFs could dictate a larger role for ATR in the absence of ATM (Cimprich and Cortez, 2008).

If ATM and DNA-PKcs are both stimulated by the same lesion (a DSB), recruited with rapid kinetics, and have similar substrate specificity, why are CSR, chromosomal integrity, apoptosis, and substrate phosphorylation more severely compromised by the loss of ATM than of DNA-PKcs, and why are the phenotypes of ATM$^{-/-}$ and DNA-PKcs$^{-/-}$ mice so different? DSBs recruit and activate DNA-PKcs and also induce rapid ATM-dependent DNA-PKcs phosphorylation, which paradoxically contributes to the dissociation of DNA-PKcs from DNA ends (Chen et al., 2007; Merkle et al., 2002; Uematsu et al., 2007). As suggested by the defects in CSR, DNA-PKcs may play an important structural role in repair (Figure 1; Figure S4). Therefore, we propose that if a lesion fails to be repaired in a timely manner by a DNA-PKcs-dependent end-joining mechanism, ATM-mediated DNA-PKcs phosphorylation would induce dissociation of DNA-PKcs from DNA ends, while at the same time triggering cell cycle arrest, apoptosis, or delayed repair (Figure S9). In this model, DNA-PKcs would only maintain its

presence at a DSB and efficiently phosphorylate DNA repair and checkpoint substrates in the absence of ATM. This switch from functioning as a structural component of the NHEJ pathway in ATM sufficient cells to a back-up signaling molecule in ATM deficient cells would explain why DNA-PKcs can have paradoxically opposite roles in the DNA damage response. For example, loss of DNA-PKcs promotes a p53-dependent checkpoint in ATM sufficient thymocytes (Guidos et al., 1996), but severely impairs p53-dependent apoptosis in ATM^{-/-} thymocytes (Figure 6).

A non-exclusive explanation for why ATM might be the "primary" DSB kinase is that ATM signaling is associated with massive signal amplification. A small number of breaks are capable of activating most of the cellular pool of ATM (Bakkenist and Kastan, 2003), whereas full DNA-PKcs activation, measured by auto-phosphorylation, seems to require higher levels of DNA damage (Douglas et al., 2007; Uematsu et al., 2007). ATM amplification is promoted by interaction with Mre11/Rad50/Nbs1 on chromatin flanking the DSB site, whereas DNA-PKcs activation seems to be limited to the DNA end bound by Ku (You et al., 2007) (Figure S9). Taken together, the combined effect of ATM mediated rapid dissociation of DNA-PKcs from the DNA break as well as amplification of ATM signaling may explain why very few *in vivo* substrates for DNA-PKcs have hitherto been discovered. Consistent with both hypotheses, aberrant ATM recruitment and or amplification in Nijmegen breakage syndrome (NBS) cells and cells expressing a C-terminally truncated Nbs1 protein results in a greater dependence on DNA-PKcs-phosphorylation of H2AX (Falck et al., 2005) and Chk2 (Figure S10) relative to normal cells.

Ataxia telangiectasia (AT) is a pleotropic disease caused by dysfunction of the ATM kinase. Clinically, the most devastating feature of AT is loss of cerebellar neurons, which is believed to be due to defects in the DNA damage response. Neurons in ATM^{-/-} mice are resistant to DNA damage induced apoptosis and there is little neuronal degeneration in mice, whereas other tissues, such as the intestines, are hypersensitive, and other cell types, such as lymphocytes, show intermediate radiation sensitivity (Herzog et al., 1998). We speculate that these variations in cellular responses to DNA damage may reflect tissue and species specific differences in the degree to which PIKK family members compensate for lack of ATM. This may reflect variations in the cell cycle status, and the abundance of DNA-PKcs in different tissues.

EXPERIMENTAL PROCEDURES

Mice

ATM^{-/-} and 53BP1^{-/-} mice were gifts from Anthony Wynshaw-Boris and Junjie Chen respectively. p53^{-/-} mice were purchased from Taconic laboratories, and Nbs1[657Δ5], Nbs1[tr735], and DNA-PKcs^{-/-}HL mice were generated as described (Difilippantonio et al., 2007; Manis et al., 2002; Zha et al., 2008). The ATM[c/c] allele was produced as described (Zha et al., 2008). The characterization of the ATM[c/c] mouse phenotype will be published elsewhere (Zha et al. in preparation).

B cell cultures, FISH detection of genomic instability and *c-myc/IgH* translocations

B cells were isolated, stimulated and assessed for CSR as described (Reina-San-Martin et al., 2003). For irradiation experiments, cells were mock or pre-treated with ATMi Ku55933 (5uM), PKi NU7026 (20uM) or PKi NU7441 (500nM) for 1 hour. For CSR and metaphases, cells were mock or pretreated with PKi NU7026 (20uM) for 72 hours. For FISH analysis, metaphases were hybridized with chromosome 12 painting, IgH Cα BAC, and telomere-repeat specific PNA probes as described (Callen et al., 2007). PCR and Southern blotting analysis of *c-myc/IgH* translocations were performed as described (Ramiro et al., 2004).

Western blotting

Western blot analysis was performed as described (McBride et al., 2006; Pellegrini et al., 2006). Primary antibodies were used at the following dilutions: anti-mouse Atm S1987p (1:400) (Pellegrini et al., 2006), anti-Smc1 S957p (1:400, Rockland Immunochemicals), anti-Smc1 (1:2,000, Novus), anti-p53 S15p (1:500, Cell Signaling), anti-p53 (1:2,000, Cell Signaling), anti-Chk1 317p (1:600, Bethyl Labs), anti-Chk2 (1:1,500, Upstate Biotechnology), anti-tubulin (1:15,000, Sigma), anti-γH2AX (1: 1,000 Upstate Biotechnology), anti-Kap-1 S824p (1:700, Bethyl Labs), anti-53BP1 S25p (0.2 μg/ml, Bethyl Laboratories) and anti-AID (1:1000) (McBride et al., 2006).

shRNA and viral infections

A series of small hairpin RNA vectors against DNAPKcs were selected from Open Biosystems, with the following sequences: Prkdc 59: GCGACATATTATGGAAGAATT; Prkdc 61:CCACCCAACAACAATATGATT; Prkdc 62: GCCATACAAATGTGGAATTAA. For experiments in MEFs, DNA fragments encoding ShRNA's were cloned into the lentiviral vector pLKO.1. For experiments in B cells DNA fragments encoding shRNAs were cloned in LMP retroviral vector (Open Biosystems). For immortalized MEF infection, ShRNA lentiviruses were added to MEFs and 24 hours later cells were selected with puromycin. Primary MEFs were isolated from mice harboring conditionally deleted alleles for the kinase domain of ATM as described in (Zha et al., 2008). ATMc/+, ATMc/c and ATMc/c DNAPK-/- cells were directly infected with a high-titer adenovirus Cre-GFP (Gift from R. C. Mulligan, Harvard Medical School). Four days after infection cells were harvested and protein lysates were prepared. B cells were activated with LPS and IL4 were infected with retrovirus carrying DNA-PKcs specific or control shRNA as described (Ramiro et al., 2006) or with a retrovirus carrying Cre recombinase as described (Robbiani et al., 2008). GFP positive cells were sorted after 5 days, irradiated and phosphorylation was measured by western blot.

Quantitative RT-PCR

Switch region transcription was measured by RT-PCR as described (Reina-San-Martin et al., 2004). Thymocytes were pre-incubated with DNA-PKcs inhibitor for 1 hr, irradiated, and incubated in media for 4 hours before RNA was extracted and reverse transcribed using a Super ScriptIII cDNA synthesis kit (Invitrogen). Quantitative RT-PCR was performed using SYBR Green (Perkin Elmer) with the 7900HT Fast Real-time PCR system (Applied Biosystems). PCR conditions were 40 cycles of 95C for 30s followed by 1 min at 61C. All samples were run in triplicate. PUMA primers are as described in (Alvarez et al., 2006). P21 primers: F5'-AAGTGTGCCGTTGTCTCTTC-3', R 5'-ACTTCAGGGTTTTCTCTTGC-3' NOXA primers: F5'-GTTGAGCACACTCGTCTTCAA-3', R5'-GTTGAGCACACTCGTCCTTCAA-3' GAPDH primers: F5'-CCAGTATGACTCCACTCACG-3' and R5'-GACTCCACGACATACTCAGC-3'. BAX primers are described in (Boley et al., 2002).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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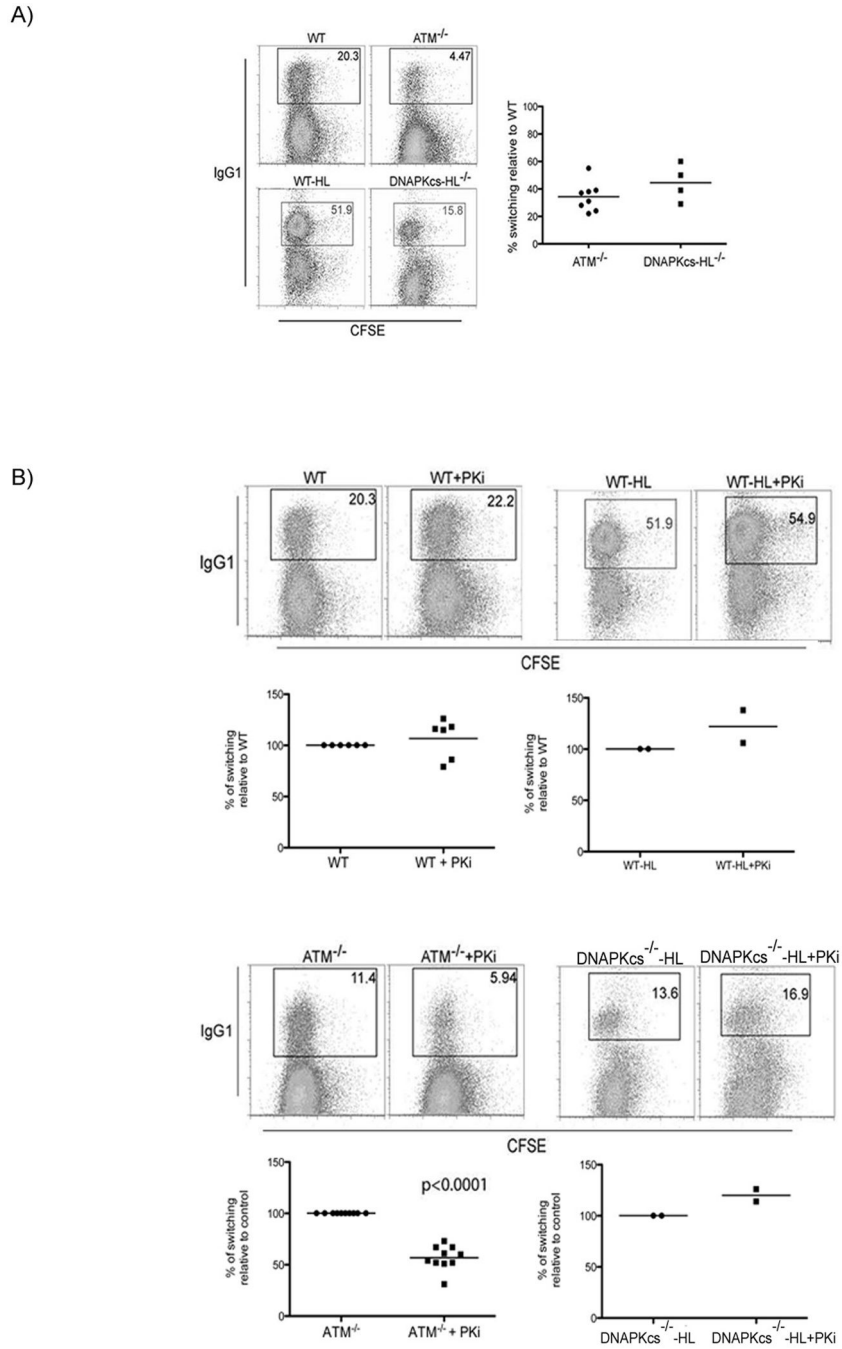
Figure 1. Contribution of DNA-PKcs holoenzyme and DNA-PKcs kinase activity to CSR
(**A**) Flow cytometric analysis of CSR to IgG1 in ATM^{-/-}, wild type (WT, littermate control for ATM), DNA-PKcs^{-/-}-HL and WT-HL (littermate control for DNA-PKcs^{-/-}-HL). DNA-PKcs^{-/-}-HL and WT-HL are matched littermates expressing immunoglobulin heavy VB1-8 and light chain V3-83 genes that rescue B cell development in DNA-PKcs^{-/-}mice (Manis et al., 2002). B cells were labeled with CFSE to assess cell division and stimulated with LPS+IL4 for 3 days. Graphs show switching relative to matched wild type sample, each dot represents an individual experiment and the bar represents the average. (**B**) Flow cytometric analysis of CSR as in (**A**) in the presence or absence of PKi NU7026. Graphs as in (**A**) but show switching relative to the matched sample in the absence of Pki as indicated.

A)

% of sequences — bp overlap (WT / WT + PKi)

% of sequences — bp overlap (ATM$^{-/-}$ / ATM$^{-/-}$ + PKi)

B)

Sμ (J00440)	MH	INSERTION	MH	Sγ1(D78344)

ATM$^{-/-}$

GACTGTAATGAACTGGAATGAGCTGGG CC   CCAGCTCAGC--TCACCCCAGCTCAGCT/CAGCTCAGCTCACCCCAACTC   AGGTGCAAGTTAAGTACTTGTAGAGGAA
Sμ5160   Sμ5188   Sμ5520   Sμ5380   Sμ5487   Sμ 5462   Sγ 2880   Sγ 2908
TGAGCCAAACTGGAATGAACTTCATTAA   CTGAACAGATAGAAG--TAGATATAGGGAATCCAGTTGAGGTGGAAGAAA   GGGG   ATCCAGGCAGAGTAGCTATAGGGCAGCC
Sμ5220   Sμ5248   Sγ 2621   Sγ 2686   Sγ 8005   Sg8033
ACTGCCTACACTGGACTGTTCTGAGCTG   CCCCAGCTCAGCCCAGCTCAGCCCAGCTCAGCCCAGCTCACCCC   AGGACAGGGAAGCTATAGGGAAACCAGG
Sμ5273   Sμ5301   Sμ5540   Sμ5497   Sγ8131   Sg8159
GCTCAGCTATGCTACGCTGTGTTGGGGT   AGCTGAGATGGGGTGAGATGGGGTGAGCT   GAGC   CAGGAGAGGTAGAATTGTGGGACTCCAG
Sμ5317   Sμ 5345   Sμ 5374   Sμ 5402   Sγ2767   Sγ 2795

ATM$^{-/-}$+PKI

TTGCCAGTAAATGTACTTCCTGCTTGTT   GAAGTGTGGTGACCC--AGTGTGTTGATCCAGACAAAACACCTACAGGGG   AGACAAATCAGCTAGATTGGGCGGATCC
Sμ4765   Sμ4793   Sγ6598   Sγ6680   Sγ7833   Sγ7861
AGCCCTAGTAAGCGAGGCTCTAAAAAGC A   CAGCTCCATGACAGGCAGGACAGGTGGAAGTGTGGTAC   CCAGGCAGAGCAGCTCCATGACAGCCAG
Sμ5026   Sμ5054   Sγ5446   Sγ5484   Sγ6708   Sγ6736
TGTTAAAGAATGGTATCAAAGGACAGTG   GTAGGAG   CAGGAGAAATGGAAGAATGCAGA-TCCA
Sμ4789   Sμ4817   Sγ8029   Sμ8057
TCTGAGCTGAGATGAGCTGGGGTGAGCT   AAATGGC   TACAGAGAAGCTGAGGCAGGTAAGAGTG
Sμ5292   Sμ5320   Sγ8418   Sγ8446
TCAATGTGGTTTAATGAATTTGAAGTTG   AAGTTGAAGAG   TGGGGATCTAGGCAGAAGTGGTACAGGG
Sμ4740   Sμ4768   Sγ2445   Sγ2473
TTCATTAATCTAGGTTGAATAGAGCTAA   CAAAACACCTACAGGGG   AGACAAATCAGCTAGATTGGGCGGATCC
Sμ5240   Sμ5268   Sγ7833   Sγ7861
GCTAAGCTGGGGTGAGCTGAGCTGAGC   TGGCTGAGCTAGGGTGAGCTGGGCTGGGGCTGGGGTGAGCTGAGCTGAGCTCACCCC   CAACTAGAAGTGTGTGAATCCAGGCAGA
Sμ5453   Sμ5481   Sμ5425   Sμ5470   Sγ8084   Sγ8112
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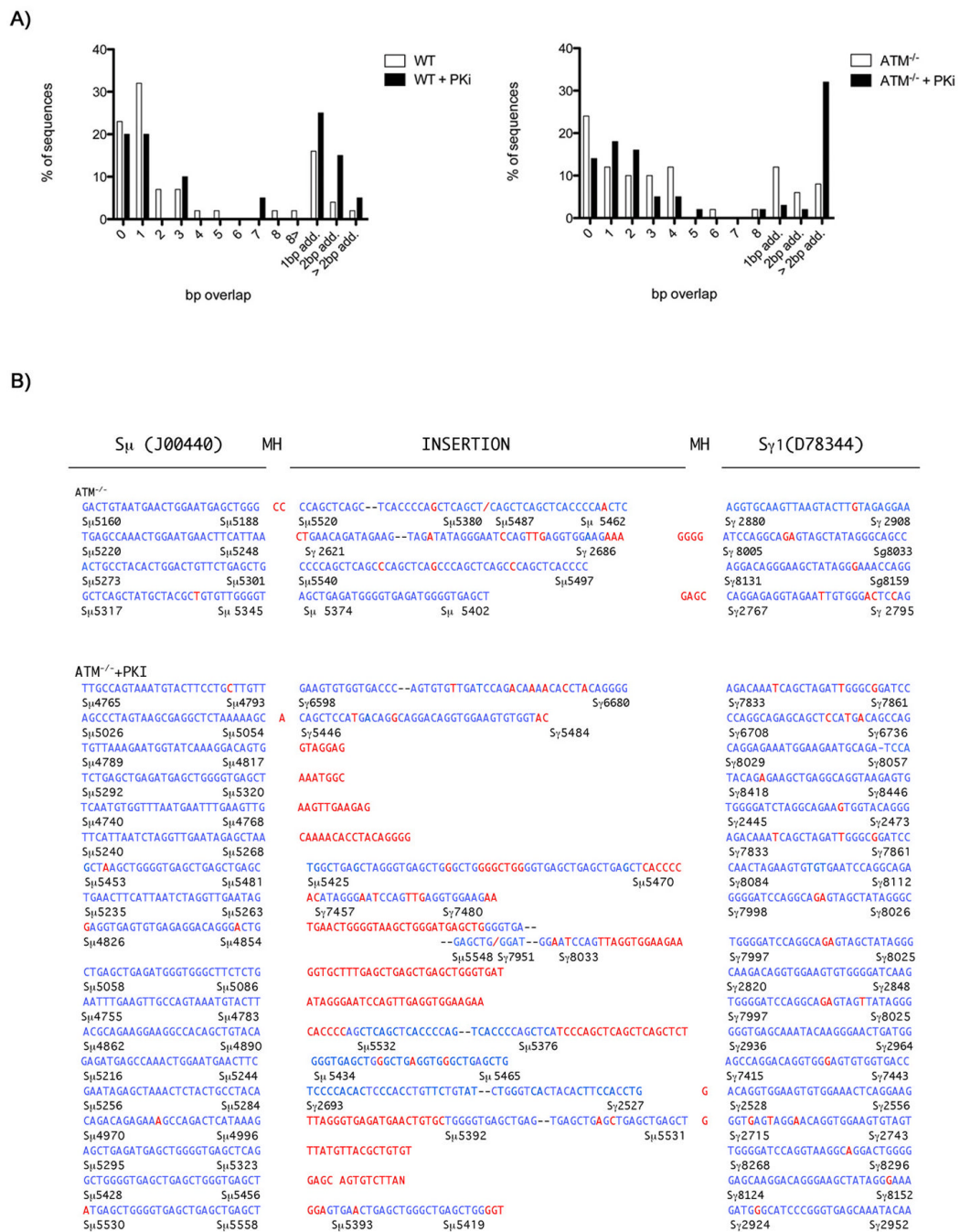Sμ5235   Sμ5263   Sγ7457   Sγ7480   Sγ7998   Sγ8026
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Sμ4826   Sμ4854   --GAGCTG/GGAT--GGAATCCAGTTAGGTGGAAGAA   TGGGGATCCAGGCAGAGTAGCTATAGGG
                                    Sμ5548 Sγ7951   Sγ8033   Sγ7997   Sγ8025
CTGAGCTGAGATGGGTGGGCTTCTCTG   GGTGCTTTGAGCTGAGCTGAGCTGGGTGAT   CAAGACAGGTGGAAGTGTGGGGATCAAG
Sμ5058   Sμ5086   Sγ2820   Sγ2848
AATTTGAAGTTGCCAGTAAATGTACTT   ATAGGGAATCCAGTTGAGGTGGAAGAA   TGGGGATCCAGGCAGAGTAGTTATAGGG
Sμ4755   Sμ4783   Sγ7997   Sγ8025
ACGCAGAAGGAAGGCCACAGCTGTACA   CACCCCAGCTCAGCTCACCCCAG--TCACCCCAGCTCATCCCAGCTCAGCTCAGCTCT   GGGTGAGCAAATACAAGGGAACTGATGG
Sμ4862   Sμ4890   Sμ5532   Sμ5376   Sγ2936   Sγ2964
GAGATGAGCCAAACTGGAATGAACTTC   GGGTGAGCTGGGCTGAGGTGGGCTGAGCTG   AGCCAGGACAGGTGGGAGTGTGGTGACC
Sμ5216   Sμ5244   Sμ 5434   Sμ 5465   Sγ7415   Sγ7443
GAATAGAGCTAAACTCTACTGCCTACA   TCCCCACACTCCCCACCTGTTCTGTAT--CTGGGTCACTACACTTCCACCTG   G   ACAGGTGGAAGTGTGGAAACTCAGGAAG
Sμ5256   Sμ5284   Sγ2693   Sγ2527   Sγ2528   Sγ2556
CAGACAGAGAAAGCCAGACTCATAAAG   TTAGGGTGAGATGAACTGTGCTGGGGTGAGCTGAG--TGAGCTGAGCTGAGCT   G   GGTGAGTAGGAACAGGTGGAAGTGTAGT
Sμ4970   Sμ4996   Sμ5392   Sμ5531   Sγ2715   Sγ2743
AGCTGAGATGAGCTGGGGTGAGCTCAG   TTATGTTACGCTGTGT   TGGGGATCCAGGTAAGGCAGGACTGGGG
Sμ5295   Sμ5323   Sγ8268   Sγ8296
GCTGGGGTGAGCTGAGCTGGGTGAGCT   GAGC AGTGTCTTAN   GAGCAAGGACAGGGAAGCTATAGGGAAA
Sμ5428   Sμ5456   Sγ8124   Sγ8152
ATGAGCTGGGGTGAGCTGAGCTGAGCT   GGAGTGAACTGAGCTGGGCTGAGCTGGGGT   GATGGGCATCCCGGGTGAGCAAATACAA
Sμ5530   Sμ5558   Sμ5393   Sμ5419   Sγ2924   Sγ2952

Figure 2. Inhibition of DNA-PKcs kinase activity increases the frequency of CSR junctions with insertions

(**A**) Wild type and ATM$^{-/-}$ B cells were either untreated or pretreated with PKi NU7026, stimulated with LPS+IL4 for 72 hours, and IgG1^{+} cells were sorted for further analysis. Genomic DNA was amplified by PCR, and Sμ/Sγ1 junctions were sequenced. Percentage of junctions with indicated nucleotide overlap or insertions is indicated. (**B**) Sμ/Sγ1 switch junctions with insertions greater than 2bp from ATM$^{-/-}$ B cells in the presence or absence of PKi NU7026. The numbers below each sequence correspond to Sμ (Genbank J00440) and Sγ1 (Genbank D78344) germline sequences. Blue letters indicate sequences homologous to germline, red letters indicate mutations, insertions with no known homology or microhomology

at junctions, -- indicates contiguous sequences that are not shown, / indicates a break in the sequence. Number of sequences analyzed: ATM$^{-/-}$=50; ATM$^{-/-}$+PKi=56; WT=44; WT +PKi=20.
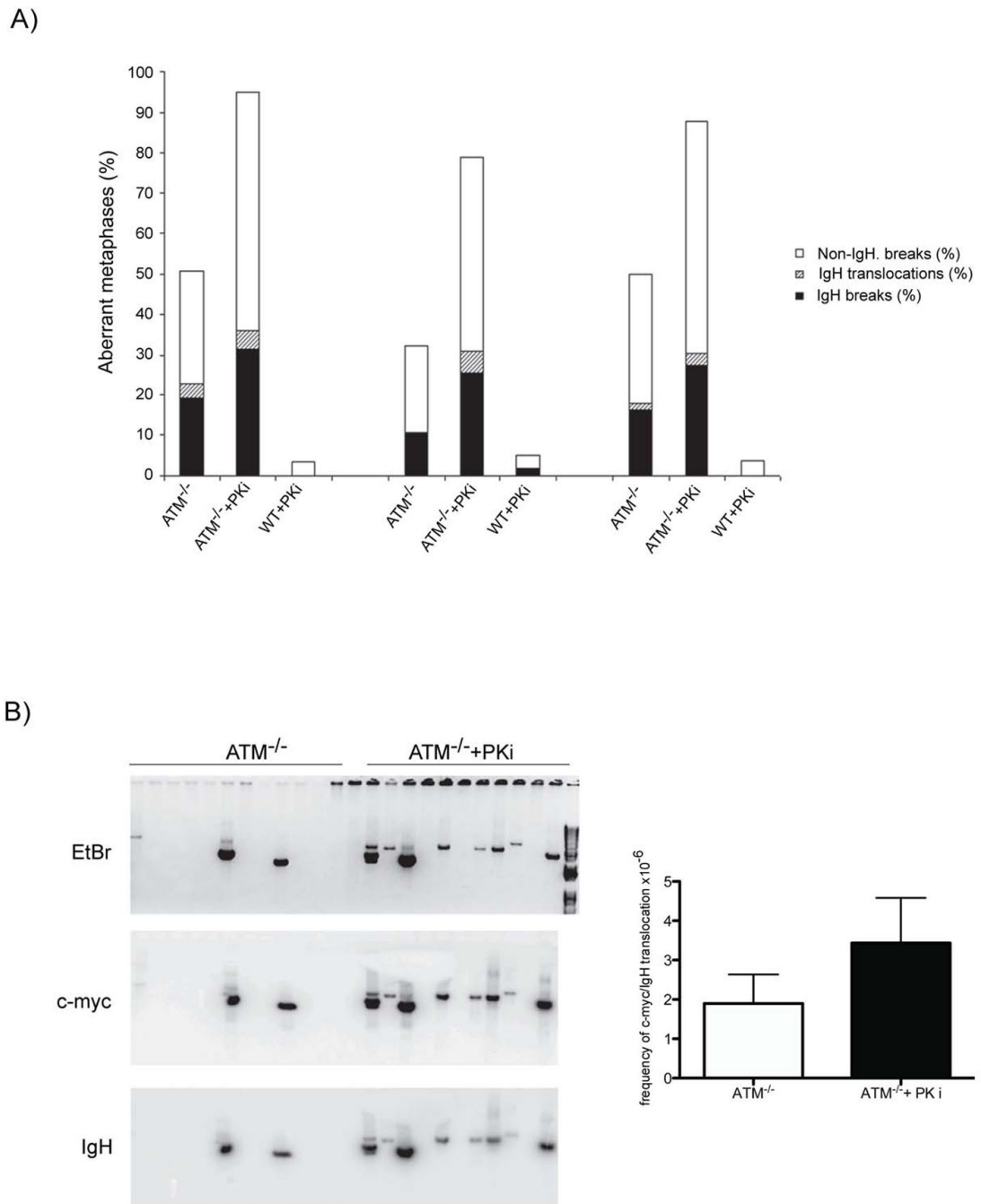
A)

B)

Figure 3. Loss of DNA-PKcs kinase activity results in a synergistic increase in genomic instability in ATM$^{-/-}$ B cells
(**A**) Frequency of IgH locus breaks (black), IgH locus translocations (grey, striped), and non-IgH breaks (white) in 3 independent cultures of ATM$^{-/-}$ and wild type B cells mock or pre-treated with PKi NU7026. Metaphase spreads (>80 analyzed for each experiment) were prepared 72 hours after B cell stimulation with LPS and IL4. (**B**) *c-myc/IgH* translocations. Representative agarose gel (top) and Southern blots with *c-myc* (middle) and *IgH* (bottom) probes. Frequency of *c-myc/IgH* translocation per 10^6 cells is plotted on the right p=0.0002 (ATM$^{-/-}$ vs. ATM$^{-/-}$ pretreated with PKi NU7026).
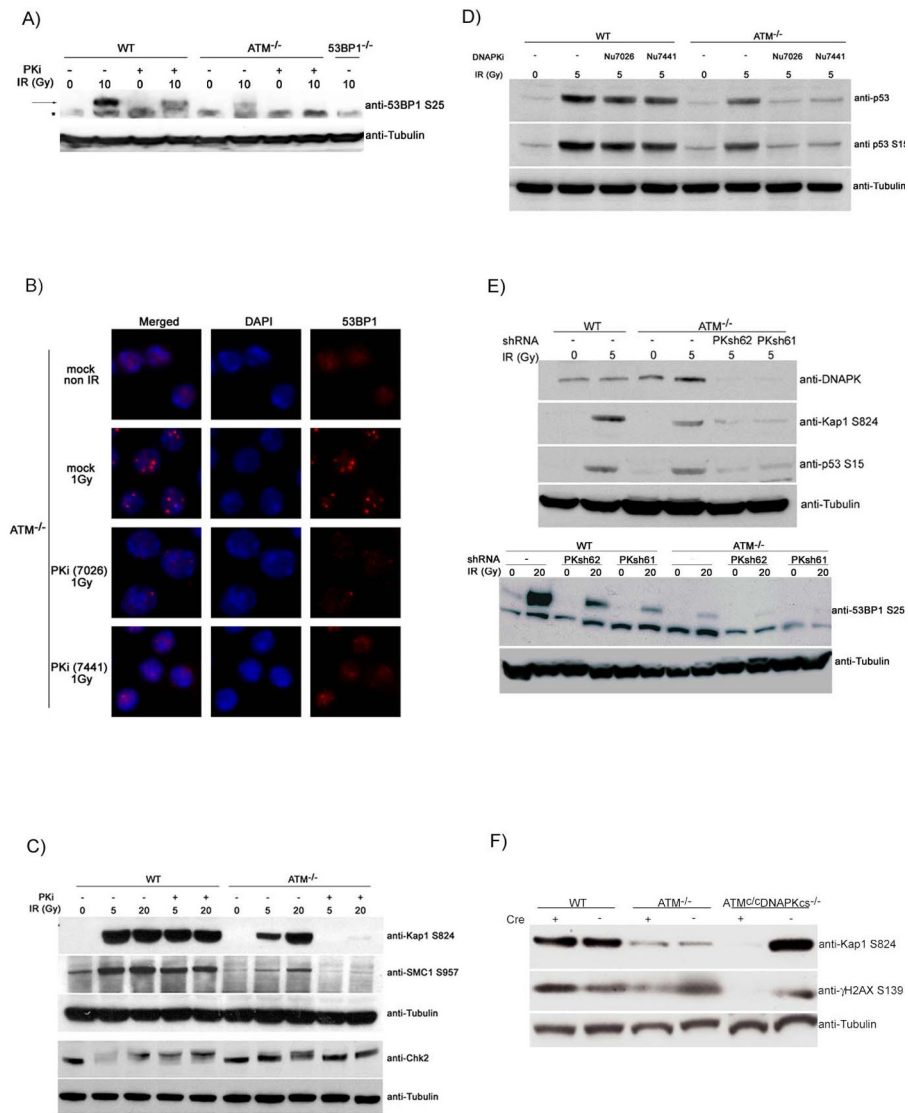
Figure 4. ATM and DNA-PKcs collaborate to phosphorylate 53BP1, Kap-1, p53, SMC1 and Chk2 in lymphocytes
(**A**) Wild type, ATM$^{-/-}$ and 53BP1$^{-/-}$ B cells were cultured with LPS and IL4 with or without PKi NU7026 for 48h, and harvested 1 hour after 0 or 10 Gy irradiation as indicated. Western blots show 53BP1 phosphorylation (arrow) and tubulin loading controls. * indicates non-specific band. (**B**) 53BP1 focus formation in ATM$^{-/-}$ B cells 6 hours after treatment with PKi (NU7026) or another potent and selective inhibitor of DNA-PKcs (NU7441) (Leahy et al., 2004) and 1Gy irradiation. (**C**) Western blots for Kap-1, SMC1, and Chk2 phosphorylation and tubulin loading controls in irradiated B cells treated as in (**A**). (**D**) Western blot shows p53 protein levels and p53 phosphorylation on Ser15, 4 hours after 0 or 5 Gy treatment. Cells were pretreated with PKi (NU7026) or an independent selective inhibitor of DNA-PKcs (NU7441). (**E**) DNAPKcs was depleted in cultured WT and ATM$^{-/-}$ B cells using short hairpin RNAs. Five days after infection, cells were untreated or irradiated, sorted and blotted for DNA-PKcs, phosphorylated Kap-1, phosphorylated p53 (upper panel) or phosphorylated 53BP1 (lower panel). (**F**) B cells from WT, ATM$^{-/-}$ and conditional ATM$^{c/c}$DNAPK$^{-/-}$ mice were infected with a retrovirus expressing Cre-GFP (+) or GFP (-) for 5 days. Cells were sorted, irradiated and levels of Kap-1 and H2AX phosphorylation were assessed by Western blotting.
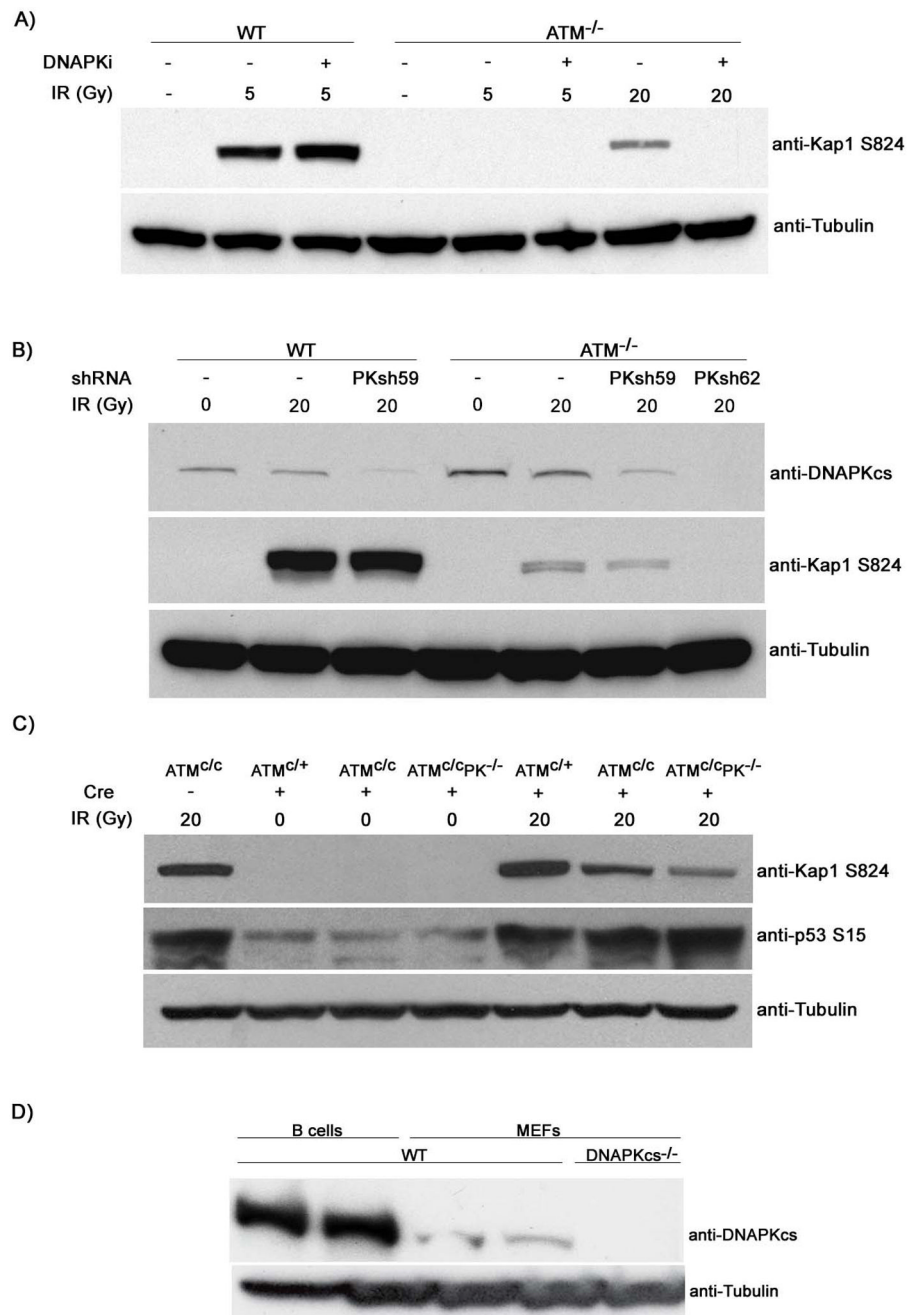
Figure 5. ATM and DNA-PKcs collaborate in IR induced phosphorylation of Kap-1 but not in p53 activation in MEFs

(**A**) Immortalized WT and ATM$^{-/-}$ MEFs were preincubated for 1hour with or without PKi and levels of phosphorylated Kap-1 were determined 1hour after 5 or 20Gy irradiation. (**B**) Levels of DNAPKcs and IR induced phosphorylated Kap-1 in immortalized WT and ATM$^{-/-}$ MEFs stably expressing lentiviruses harboring PKcs shRNA. Down regulation of DNA-PKcs in ATM$^{-/-}$ MEFs was more efficient using PKsh62 compared to PKsh59. (**C**) Passage 2 primary MEFs derived from ATM$^{c/+}$, ATM$^{c/c}$ or ATM$^{c/c}$DNAPKcs$^{-/-}$ mice were infected with an adenovirus expressing Cre-GFP (+), irradiated with 20Gy and levels of Kap-1 and p53 phosphorylation were analyzed by Western blotting 1 hour post irradiation. (**D**) Decreased levels of DNA-PKcs in WT MEFs relative to B cells as determined by Western blotting.
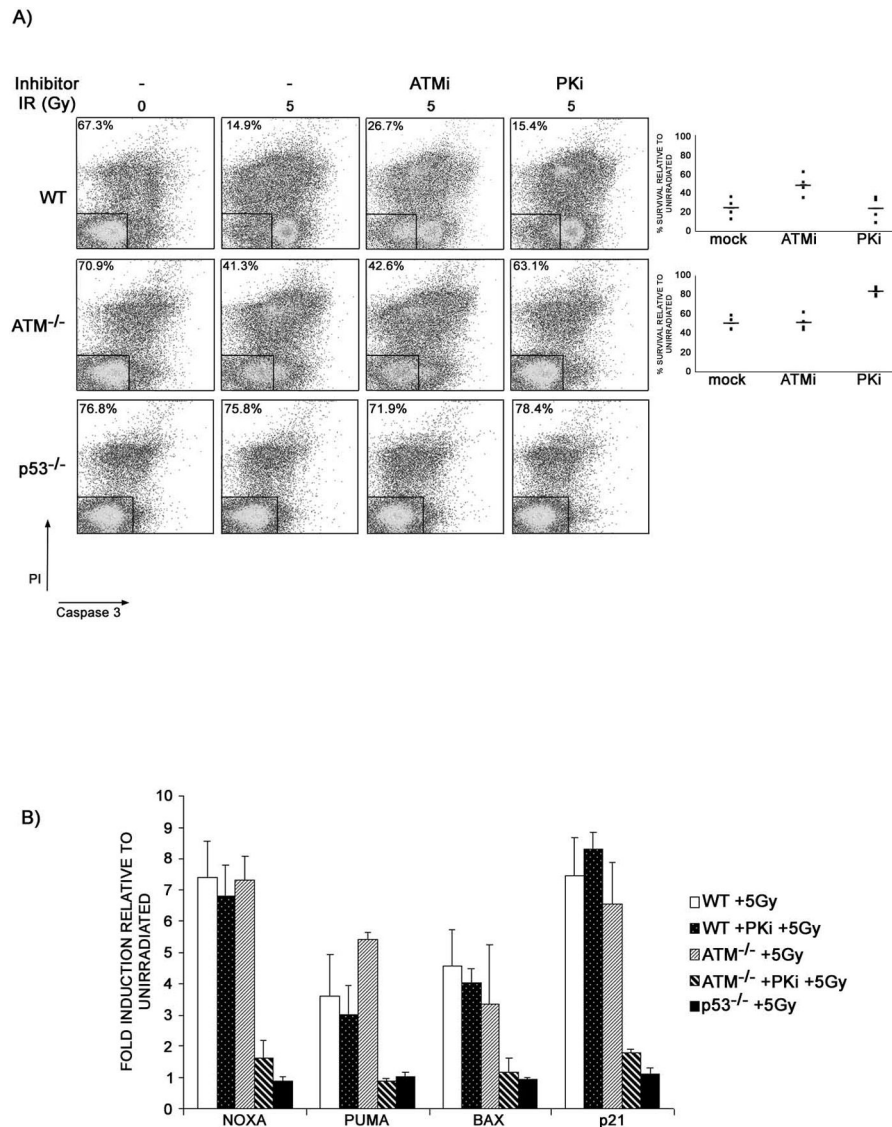
A)

B)

Figure 6. ATM and DNA-PKcs collaborate in IR induced p53 dependent apoptosis
(**A**) Wild type, ATM$^{-/-}$, and p53$^{-/-}$ thymocytes were mock or pre-treated with ATMi (KU55933) or PKi (NU7026), irradiated with 5 Gy and the percentage of live cells was measured by flow-cytometry 24 hours post-IR using a caspase-3-substrate reagent (OncoImmunin Inc.). Percentage of live cells (Propidium Iodide (PI) and Caspase 3 negative) is indicated. Graph shows results of individual experiments (dots) normalized to unirradiated drug-treated samples of the same genotype. Bars indicate the average. (**B**) Quantitative RT-PCR analysis of p53-dependent induction of NOXA, PUMA, BAX and p21 mRNAs 4 hours after treatment with 5Gy plotted relative to un-irradiated controls of the same genotype. An average of two experiments performed in triplicate is shown. The experiments were repeated 6 times with similar results.