Double-strand break (DSB) recognition is the first step in the DSB damage response and involves activation of ATM and phosphorylation of targets such as p53 to trigger cell cycle arrest, DNA repair or apoptosis. It was reported that activation of ATR kinase by DSBs also occurs in an ATM-dependent manner. On the other hand, Ku70/80 is known to participate at a later time point of the DSB response, recruiting DNA-PKcs to facilitate non-homologous end joining. Since Ku70/80 has a high affinity for broken DNA ends and is abundant in nuclei, we examined their possible involvement in other aspects of the DSB damage response, particularly in modulating the activity of ATM and other PI-3 related kinases during DSB recognition. We thus analyzed p53ser18 phosphorylation in irradiated Ku-deficient cells and observed persistent phosphorylation in these cells relative to wild type cells. ATM or ATR inhibition revealed that this phosphorylation is mainly mediated by ATM-dependent ATR activity at 2 hours post-IR in wild type cells; while in Ku-deficient cells, this occurs mainly through direct ATM activity, with a secondary contribution from ATR via a novel ATM-independent mechanism. Using ATM/Ku70 double null cell lines which we generated, we confirmed that ATM-independent ATR activity contributed to persistent phosphorylation of p53ser18 in Ku-deficient cells at 12 hours post-IR. In summary, we discovered a novel role for Ku70/80 in modulating ATM-dependent ATR activation during DSB damage response and demonstrated that these proteins confer a protective effect against ATM-independent ATR activation at later stages of the DSB damage response.
phosphorylation of these proteins is diminished or delayed; the residual phosphorylation is thought to be mediated by the ATM- and Rad3-related (ATR) kinase, which is another member of the PI-3 related kinase family (11). It has recently been revealed that the activation of ATR by DSBs is also ATM-dependent (12-15).

Current knowledge assigns a role for the Ku70/80 heterodimer in the later stages of the DNA damage response, particularly in one of the two processes for DSB repair, non-homologous end joining (NHEJ) (16,17). In this process, the Ku70/80 heterodimer binds to the free DNA ends at a DSB, and recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), the third member of the PI-3 related kinase family. The formation of the DNA-PK complex at the site of the DSBs results in the recruitment and phosphorylation of XRCC4, DNA Ligase IV, Cernunnos/XLF, and Artemis to ligate the broken DNA ends (18-22).

Since Ku70/80 possess a high affinity for DNA broken ends and is highly abundant in the nucleus, we were interested in determining whether these proteins might also influence the signaling aspects of the DNA damage response, particularly the activation of ATM and other PI-3 related kinases during initial DSB recognition. To explore this possibility, we performed PI-3 kinase inhibition in Ku70 and Ku80-deficient cells and then examined the phosphorylation of p53ser18 following irradiation. Furthermore, we successfully established Ku70 and ATM double deficient cells and used these cells to analyze the possible relation of the Ku70/80 heterodimer with ATM and ATR signaling in the early stages of the DSB response. Our findings suggest a novel role for the Ku70/80 heterodimer in the early stages of the DNA damage response, particularly, in modulating ATM-dependent ATR activation in response to DSB damage. Moreover, we have proven the existence of an ATM-independent mechanism for ATR activation following DSB damage in Ku-deficient cells.

MATERIALS AND METHODS

Cell Culture and Induction of DNA damage- The following spontaneously immortalized fibroblast cell lines were derived from mice with genotypes indicate parentheses: PK34N (wild type), PK33N (DNA-PKcs) (23), PK/80-1A (DNA-PKcs Ku80) (23,24), A82-1 (ATM) (25), D14-3 (ATM Ku702loxP/2loxP). Only STEFKu70 (Ku70) (26) is a Papiloma virus E6, E7-transformed mouse fibroblast cell line. These cells were maintained in a humidified atmosphere with 5% CO2 in DMEM containing 5% calf serum, supplemented with penicillin and streptomycin. Cells were grown to about 70-90% confluence in 12 well plates and were irradiated with X-rays at the rate of 1.0Gy/min (150kV, 5mA) (MBR-1505R2, Hitachi Medico, Japan) to achieve a cumulative dose of 8Gy for all experiments unless otherwise mentioned.

PI-3 related kinase inhibition experiments- Cells were incubated with indicated concentrations of Wortmannin (Sigma, USA), caffeine (Kanto Chemical, Japan) or KU55933 for one hour and then treated with 8Gy of IR. After 2 and 12 hours, cell extracts were prepared for western blotting. Wortmannin and KU55933 were dissolved in DMSO at 10mM and 1mM respectively as a stock solution. Caffeine was dissolved in water at a concentration of 100mM.

Protein extraction and western blotting- Western blotting experiments were performed using whole cell extracts following standard techniques. Cells were resuspended and lysed in 1X SDS buffer (67.5mM Tris pH6.8, 25mM NaCl, 0.5mM EDTA, 12.5% Glycerol, 2.5% SDS, 100mM DTT). Lysates were boiled for 2 minutes, and sonicated. For western blotting, cell extracts were electrophoresed on 8% SDS-polyacrylamide gels to detect medium-sized proteins or low-bis 8% SDS-acrylamide gels for high molecular weight proteins. Proteins were then transferred to a PVDF membrane (GE Healthcare, UK). Membranes were incubated in TBS-T (137mM NaCl, 2.7mM KCl, 25mM Tris pH7.4 and 0.1% Tween-20) and 5% skim milk (Snow Brand, Japan) added to TBS-T. Membranes were then stained with Ponceau S dye to check for equal loading and homogeneous transfer.

Primary antibodies used in this study were Ku70 (C-19, M-19), ATM (2C1), ATR (M-19) (Santa Cruz Biotechnology, USA), phospho-p53 ser18 (Cell Signaling Technology, USA) and alpha-tubulin (ICN, USA). Anti-mouse and anti-rabbit secondary antibodies were obtained from GE Healthcare, and an anti-goat antibody was obtained from Jackson ImmunoResearch (USA). Proteins were visualized using ECL western Blotting Detection Systems (GE Healthcare). After probing with the phospho-specific
antibodies, immunoblots were stripped and reprobed with tubulin to check for equal loading.

siRNA transfections- All siRNA transfections were performed using Lipofectamine 2000 (Invitrogen, UK) following manufacturer’s recommendations. Approximately 0.3-0.6X10^5 cells per well were seeded in 12-well plates with 1ml of antibiotic-free DMEM with 5% calf serum. The next day, cells were treated with Lipofectamine 2000 and 20pmol of control, ATR or ATM siRNA (from Qiagen; siRNA sequences available upon request). After 24 hours, this procedure was repeated. The cells were analyzed 48 hours after the last siRNA transfection.

Selective targeting of Ku70- We initially generated a tetracycline-inducible Ku70 conditional allele (Supplementary Fig.2a). Ku70 sequences were either directly derived or amplified from genomic DNA obtained from CJ7 ES cells or a cosmid clone carrying the Ku70 locus. The 5’ end of the targeting vector consisted of a 1.2 kb region possessing homology to intron 1 and was generated by high fidelity PCR (26). The early part of exon 2 containing the untranslated region (referred to as exon 2x) was fused to the tetracycline transactivator gene, tTA, having a terminal codon and poly-A sequence. The later half of exon 2 (referred to as exon 2y) beginning from the ATG start site of Ku70 was placed under the control of the tetracycline-responsive promoter, TRE. A PGK-driven neomycin selection marker was positioned between the first loxP site and the TRE-2y region. The 3’ arm of the targeting vector consisted of a 7.7 kb EcoRI fragment derived from the region spanning introns 2-5.

Generation of Ku70 conditional knockout mice- A correctly targeted ES cell clone, confirmed by Southern blot analysis, was injected into 3.5 dpc C57BL/6J blastocysts. Approximately ten ES cells were injected per blastocyst and twenty blastocysts were transferred to each pseudopregnant recipient. The resulting chimeric offspring were crossed with 40 mice to generate F1 progeny. To generate ATM deficient/Ku70 conditional mice, we crossed Ku70 heterozygotes with ATM heterozygotes (25). The resulting Ku70/ATM double heterozygotes were crossed with each other. Progeny that had an ATM^-/- Ku70^Loxp+/+ genotype were identified by PCR screening. Primary fibroblast cells were obtained from one of the ATM homozygous null, Ku70 homozygous conditional (ATM^-/- Ku70^Loxp+/+), mice, D14-3 and further cultured to obtain a spontaneously transformed cell line.

Results

Prolonged phosphorylation of p53ser18 in Ku-deficient cells- The most well known ATM and ATR substrate is the tumor suppressor protein, p53 (28,29). This protein plays a major role in cellular responses to DNA damage and other genomic aberrations (30). Activation of p53 leads to either cell cycle arrest to allow DNA repair or apoptosis. DSBs induce ATM-dependent phosphorylation of p53ser18 and results in reduced interaction of p53 with its negative regulator, oncoprotein MDM2 (31).

To investigate if the Ku70/80 status of a cell might influence DNA damage signaling, we examined p53ser18 phosphorylation and response to IR over a time course. We utilized four fibroblast cell lines; PK34N (wild type), and three other cell lines that were null mutants for components of the DNA-PK complex namely: PK33N (DNA-PKcs^-/-) (23), STEFKu70 (Ku70^-/-) (26), PK/80-1A (DNA-PKcs^-/-Ku80^-/-), and PK/80-193A (DNA-PKcs^-/-Ku80^-/-) (23,24) . Cells were either mock-treated or subjected to 8Gy of IR, and harvested at indicated time points. Processed samples were analyzed for p53ser18 phosphorylation by western blotting, with tubulin as a loading control (Fig.1).

Ku70-deficient mice exhibit decreased levels of Ku80 while Ku80-deficient mice show decreased...
levels of Ku70, revealing the functional synergy of these proteins (24). Previous studies have shown that Ku70 and Ku80-deficient cells have similar phenotypes. Thus, when discussed together, we shall collectively refer to these three cell lines as Ku-deficient cells.

In wild type cells and DNA-PKcs-deficient cells, phosphorylation of p53ser18 was transient and reached maximum levels 2 hours after irradiation. At 8 hours post-IR, phosphorylation levels dropped significantly with p53ser18 levels returning to background levels at 12 hours post-IR. On the other hand, phosphorylation of p53ser18 was persistent, with minimal change within the 16 hour time period after IR for both Ku80 and Ku70-deficient cells.

Our results indicate that loss of DNA-PKcs does not result in aberrant p53ser18 phosphorylation. However, loss of Ku results in persistent p53 phosphorylation. In the absence of Ku, further loss of DNA-PKcs function does not enhance the persistent p53ser18 phosphorylation.

Relative contribution of ATM and ATR kinases to p53ser18 phosphorylation in Ku-deficient cell - Following DSB damage, ATM is activated to phosphorylate target proteins like p53ser18. It is currently thought that activation of ATR following DSB damage requires ATM activity (ATM-dependent ATR activation) (12-14). To determine whether ATM and/or ATR kinase(s) were involved in the persistent phosphorylation of p53ser18 in Ku-deficient cells, we first performed knockdown experiments using siRNAs. PK34N, STEFKu70, PK/80-1A and PK/80-193A cells were individually transfected with siRNAs against ATR (ATR1 or ATR3) or ATM (ATM1).

As shown in Fig.2, in wild type PK34N cells, ATR knockdown resulted in loss of p53ser18 phosphorylation, indicating that ATR activity significantly contributes to the phosphorylation of p53ser18. Moreover, although ATR activity remained intact in these cells, knockdown of ATM completely abolished p53ser18 phosphorylation at 2 hours post-IR. These results demonstrate that ATR is activated under the control of ATM, that is, in the absence of functional ATM, ATR is unable to rescue p53ser18 phosphorylation. These findings agree with the ATM-dependent ATR activation model for DSB damage response, wherein activation of ATR by ATM is required for ATR to phosphorylate its targets (13,15). Our results suggest that in the earlier stages (2 hours) of the DSB damage response, phosphorylation of p53ser18 is predominantly mediated by ATM-dependent ATR kinase activity. At 12 hours post-IR, PK34N cells treated with either ATR or ATM siRNA exhibited basal phosphorylation levels of p53ser18, comparable to that of cells treated with control siRNA.

Interestingly, in Ku-deficient cells, knockdown of ATR did not significantly decrease p53ser18 phosphorylation at 2 hours post-IR. On the other hand, ATM knockdown resulted in a substantial reduction in p53ser18 phosphorylation. These results suggest that in the absence of Ku70/80, ATM contributes significantly to p53ser18 phosphorylation. Nevertheless, ATR participates in p53ser18 phosphorylation to a minor extent. In contrast to the predominance of ATM-dependent ATR activity observed in wild type cells, contribution of this mechanism in p53ser18 phosphorylation is minimal in Ku-deficient cells. The involvement of DNA-PKcs in phosphorylation of p53ser18 could be excluded since phosphorylation of p53ser18 was still observable in the Ku80/DNA-PKcs double deficient cells (PK/80-193A). This leads us to the question of, what kinase phosphorylates p53ser18 in the absence of ATM activity? With these results, we are led to conclude that residual p53ser18 phosphorylation after ATM knockdown is mediated by ATR through an ATM-independent mechanism.

As expected, p53ser18 phosphorylation was no longer visible at the 12 hour time point in wild type cells. The non-responsiveness of p53ser18 phosphorylation to ATM or ATR siRNA treatment reflects the return of ATM and ATR activity to basal levels at the later stage of the DSB response. For the PK/80-1A cell line, p53ser18 phosphorylation patterns in response to ATR or ATM siRNA treatment was similar to that observed at 2 hours post-IR. In the case of STEFKu70 and PK/80-193A cell lines, ATR or ATM siRNA treatment resulted in a slight reduction of p53ser18 phosphorylation levels. This suggests the involvement of ATM in p53ser18 phosphorylation and at the same time, an increase in the contribution of ATR in this phosphorylation. From these findings, we conclude that the persistent activation of p53ser18 in Ku-deficient cells is mediated by aberrant activation of ATR via an ATM-independent mechanism.

To confirm these results, we subjected cells to...
various inhibitors of PI3-related kinases. Wortmannin inhibits PI-3 kinase activity in a dose-dependent manner. Low concentrations mainly suppress ATM and DNA-PKcs, while higher concentrations are required for ATR inhibition (32-34). In wild type cells, phosphorylation of p53ser18 at 2 hours post-IR was strongly suppressed by 10µM Wortmannin (Fig.3a). In agreement with the results from our previous siRNA experiments, inhibition of ATM in wild type PK34N cells completely abolished p53ser18 phosphorylation at 2 hours post-IR. Again, we have demonstrated that p53ser18 phosphorylation is mainly mediated by direct ATM activity or through a two-step ATM-dependent process involving ATR.

At the same time point in Ku-deficient cells, some p53ser18 phosphorylation was still observed at low Wortmannin concentrations that selectively inhibit ATM, suggesting that residual p53 phosphorylation is attributed to ATM-independent ATR activity. Complete suppression of this phosphorylation was achieved at higher concentrations known to inhibit ATR activity.

In Ku-deficient cells, at 12 hours post-IR, the persistent phosphorylation of p53ser18 was difficult to suppress at low concentrations of Wortmannin. These results are consistent with our previous findings and suggest that p53ser18 phosphorylation is primarily mediated by ATM kinase activity, with a minor contribution from ATM-independent ATR activity.

Our siRNA results point to the existence of ATM-independent ATR activity in both the early and later stages of the DSB response in Ku-deficient cells. To confirm this, we also treated cells with Ku55933, a potent and specific inhibitor of ATM (IC50 for ATM is 12.9nM while IC50 for ATR is >100µM) (35,36)(Fig.3b). At the 2-hour time point, phosphorylation in wild type cells was significantly reduced by treatment with 1µM Ku55933. On the other hand, the phosphorylation in Ku-deficient cells was more resistant to Ku55933 until concentrations of up to 10µM. At 12 hours post-IR, p53ser18 phosphorylation was no longer observable in wild type cells. The 2 and 12-hour time points exhibited similar patterns of reductions in p53ser18 phosphorylation in Ku-deficient cells. Similar to the siRNA and Wortmannin experiments, we observed residual p53ser18 phosphorylation even after ATM-specific inhibition. This remaining p53ser18 phosphorylation could only reflect ATR activity that occurs independently of ATM, thus supporting the existence of ATM-independent ATR activity in Ku-deficient cells.

We also performed caffeine treatment at concentrations that inhibited both ATM and ATR activity (37-39) (Supplementary Fig.1). Wild type cells at 2 hours post-IR, and Ku-deficient cells at 2 and 12 hours post-IR, exhibited similar patterns of decrease in p53ser18 phosphorylation.

To summarize, we have demonstrated that [1] in wild type cells, at the earlier stages of the DSB damage response, phosphorylation of p53ser18 is predominantly mediated by ATM-dependent ATR activity; and [2] in Ku-deficient cells, the ATM-dependence of ATR activity is abolished at the earlier stages of the DSB response. Moreover, at the later stages, persistent phosphorylation of p53ser18 in Ku-deficient cells is mediated by both ATM activity and ATM-independent ATR activity.

Establishment of stable ATM/Ku70 double deficient cell lines- We have shown previously that wild type and Ku-deficient cells utilized differing mechanisms for phosphorylation of p53ser18. To further confirm ATM-independent ATR activation in response to DSBs in Ku-deficient cells, we generated ATM/Ku70 double deficient cells, in which ATM cannot induce ATM-dependent ATR activity.

Simultaneous knockout of ATM and Ku80 results in embryonic lethality (40). We therefore expected that knockout mice of both ATM and Ku70 would result in the same phenotype. To circumvent this difficulty, we attempted to establish transformed ATM and Ku70 double deficient cell lines from an ATM deficient Ku70 conditional cell line (Refer to materials and methods for details on the construction of these cell lines).

Using this approach, we successfully established two independent ATM/Ku70 double null cell lines, D14-3Fx1 and D14-3Fx2. Western blotting confirmed the absence of ATM and Ku70 in these cell lines (Fig.4a). They grew very poorly in regular medium but could still be expanded to generate sufficient cells for the experiments in this study.

In the ATM deficient cell lines A82-1 and D14-3, phosphorylation of p53ser18 was not observable at one hour post-IR. In contrast, ATM/Ku70 double deficient cells (D14-3Fx1 and Fx2) exhibited phosphorylation of p53ser18 at this time point, recapitulating the results from previous
ATM inhibition experiments in Ku-deficient cells.

**Persistent p53ser18 phosphorylation in ATM/Ku70 double deficient cells** - We reasoned that if ATM-independent activity existed in ATM/Ku70 double deficient cells, then we should be able to observe phosphorylation of p53ser18 in the absence of ATM kinase. We thus analyzed phosphorylation of p53ser18 in response to IR over a time course in ATM single (D14-3) and ATM/Ku70 double deficient (D14-3Fx1, D14-3Fx2) cells that were subjected to 8 Gy of ionizing radiation (Fig.4b). In wild type cells, p53ser18 was observable within one hour post-IR. Phosphorylation levels peaked at 4 hours and returned to basal levels starting at 8 hours post-IR.

In the ATM/Ku70 double deficient cell lines, D14-3Fx1 and Fx2, p53ser18 phosphorylation was still evident, clearly proving the existence of ATM-independent kinase activity towards p53ser18. More interestingly, phosphorylation levels rose slowly, reaching maximum levels at 4 hours post-IR. In contrast to the return to basal levels at 8 hours post-IR in wild type cells, significant levels of p53ser18 phosphorylation persisted beyond this time point in the ATM/Ku70 double deficient cells.

*ATM-independent ATR activity mediates phosphorylation of p53ser18 in ATM/Ku70 double deficient cells* - The next question we addressed was what kinase phosphorylates p53ser18 in ATM/Ku70 double deficient cells. We have shown in our earlier experiments that DNA-PKcs activity was not required for this event. Thus, we reasoned that the most likely candidate was ATR kinase. To further explore this possibility, we performed knockdown of ATR in ATM/Ku70 deficient cells by siRNA (Fig.5a), and also utilized the PI-3 kinase inhibitor, caffeine (Fig.5b). Because of high toxicity to D14-3Fx1 and Fx2, we could not use Wortmannin for inhibition experiments involving these cell lines.

We transfected two independent siRNAs against ATR as described in the previous experiments. In the ATM/Ku70 double deficient cells, ATR expression and p53ser18 phosphorylation were concordantly suppressed (Fig.5a). These results indicate that ATM-independent ATR activity mediated the phosphorylation of p53ser18 in ATM/Ku70 double deficient cells.

We then confirmed these findings using caffeine. Wild type, D14-3, D14-3Fx1, and D14-3Fx2 cells were treated with the indicated concentrations of caffeine, irradiated and harvested 2 or 12 hours post-IR. Wild type and ATM/Ku70 double deficient cells showed similar reduction of p53ser18 phosphorylation by caffeine inhibition at 2 hours post-IR. At 12 hours post-IR, 1mM caffeine was sufficient to significantly inhibit phosphorylation of p53ser18 in the ATM/Ku70 double deficient cells (Fig.5b). Taken together, our results from Ku single deficient cells and ATM/Ku70 double deficient cells support the existence of ATM-independent ATR activity in the Ku-deficient state.

**DISCUSSION**

In this study, we explored the involvement of the Ku70/80 heterodimer in DNA DSB damage signaling. We focused on how the Ku70/80 heterodimer might influence the activities of ATM and ATR kinases. To accomplish this, we made use of a phosphorylation site that is a target of both ATM and ATR kinases and of which phosphospecific antibodies were available, hence our choice of p53ser18 (28,29). Firstly, we have shown that IR-induced p53ser18 phosphorylation is persistent in Ku-deficient cells but not in DNA-PKcs-/- cells, suggesting that Ku70/80 may have a role in the regulation of the initial stages of DNA damage sensing. We were particularly interested in determining which of the two signaling kinases, ATM or ATR, was involved in the persistent phosphorylation of p53 in Ku-deficient cells.

It is well-established that p53ser18 is phosphorylated by ATM, ATR and DNA-PKcs (1,41). Nevertheless, we have demonstrated that the persistent phosphorylation in Ku-deficient cells does not involve DNA-PKcs activity, as we did not observe any remarkable difference between p53ser18 phosphorylation in Ku80-deficient/DNA-PKcs heterozygous cell line and the Ku80-deficient/DNA-PKcs homozygous null cell line. This finding is consistent with the fact that DNA-PKcs cannot undergo activation in Ku-deficient cells since DNA-PKcs is activated by the Ku70/80 heterodimer after their direct binding to the DNA broken ends (42).

To determine what specific kinase phosphorylated p53ser18 at 2 hours (earlier stage of IR-response) or 12 hours (later stage of IR-response) in Ku-deficient cells, we suppressed ATM or ATR...
activity by siRNA or the use of PI-3 related kinase inhibitors. From the results of ATM and ATR knockdown experiments in wild type cells at the 2 hour time point, we conclude that the predominant mechanism for p53ser18 phosphorylation is by an ATM-dependent ATR activity and not by direct ATM activity (Fig.6b). We speculate that at a very early time period such as 10-30 minutes after irradiation, ATM is the only kinase that phosphorylates p53ser18 (12)(Fig.6a). Soon after, ATR activation by ATM becomes the predominant mechanism for p53ser18 phosphorylation. At 12 hours post-IR, phosphorylation of p53ser18 was undetectable, indicating that both ATM and ATR activities have returned to basal levels (Fig.6c).

Normally, ionizing radiation induces ATM kinase activity, and ATM mainly phosphorylates p53ser18. It is well-established that Chk1 is phosphorylated by ATR in response to UV damage (43,44). However, a recent study revealed that Chk1 is also rapidly phosphorylated in response to IR in an ATR-dependent manner and that ATM-dependent ATR activity is involved in DSBs recognition in S and G2/M phase cells (14), and also in G1 phase cells (12). It is currently thought that ATM and NBS1 promote DSB-induced ATR-dependent Chk1 phosphorylation by regulating the formation of RPA-coated ssDNA that is required for ATR recruitment to sites of DNA damage (15). The cells used in this study were not synchronized, yet we have observed clear ATR activation. Thus we speculate that ATM-dependent ATR activity in response to DSBs is not restricted to S and G2/M phase but may occur at any stage of the cell cycle.

In Ku-deficient cells at 2 and 12 hours post-IR, p53ser18 phosphorylation occurs mainly through ATM; with a secondary contribution from ATR via a novel ATM-independent mechanism (Fig.6d). Moreover, we envision two possible scenarios at 12 hours post-IR; Possibility A is similar to that occurring at 2 hours post-IR while Possibility B has three kinase activities, namely: direct ATM activity, ATM-dependent ATR activity and ATM-independent ATR activity, simultaneously phosphorylating p53ser18 (Fig.6e). It was previously observed that in Ku80-deficient cells, ATM activity was increased in S phase cells (45), and ATM-independent G2 checkpoint was enhanced via Chk1 activation (46). Our results of increased ATM activity and ATM-independent ATR activation in Ku-deficient cells can well explain these observations. We have shown a novel function for Ku whereby, in the unperturbed state, Ku70/80 might modulate the process of ATM-dependent ATR activation. We envision two possibilities: [1] that Ku70/80 binds to DNA damage ends, resulting in the release of ATM from the DNA-ends and the subsequent activation of ATR at the vicinity of DNA-ends (13,45), or [2] through a mechanism similar to activation of DNA-PKcs by the Ku70/80 heterodimer, Ku70/80 activates ATR through an ATM-dependent manner (47).

Moreover, data from the ATM/Ku70 double deficient cells that we generated suggests that Ku may have a protective effect against ATM-independent ATR activation. As to why this process occurs in the event of loss of Ku function, we can conceive of two possibilities: [1] that in the absence Ku70/80, MRN remains bound to damaged DNA-ends, allowing MRN to activate ATR by its C-terminal conserved motif (47-49) or [2] broken DNA ends are left unprotected, activating exonucleases that generate single strand DNA damage, which in turn lead to activation of ATR in an ATM-independent manner (14).

In summary, we have discovered a novel role for the Ku heterodimer in the early stages of the DNA damage response, particularly, in modulating ATM-dependent ATR activation in response to DSB damage. Moreover, we have proven the existence of an ATM-independent mechanism for ATR activation following DSB damage in Ku-deficient cells. This most likely occurs as a back-up mechanism in the event of inadequacy of the DSB response machinery, such as during loss of Ku function. Whether MRN and Ku70/80 synergistically or exclusively bind to the DNA ends is yet to be known. Further studies on the interactions of Ku70/80 with ATM and ATR and their substrates are of importance to achieve a deeper understanding of the mechanisms involved in the DNA damage response.
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FIGURE LEGENDS

**Fig.1** Ionizing radiation-induced phosphorylation of p53 is persistent in Ku-deficient cells. PK34N (wild type), PK33N (DNA-PKcs−/−), STEFKu70 (Ku70−/−), PK/80-1A (DNA-PKcs−/−Ku80−/−), PK/80-193A (DNA-PKcs−/−Ku80−/−) cell lines were exposed to 8 Gy of IR and harvested at indicated time points. Phosphorylation of p53ser18 was then analyzed by western blotting. Equal loading was confirmed using alpha-tubulin antibody. Images shown are representative data from three independent experiments.

**Fig.2** Inhibition of ATR or ATM kinase by siRNA attenuates IR-induced p53ser18 phosphorylation. Cells were transfected with small inhibitory RNA against ATR or ATM kinases. Two independent siRNA sequences (ATR1 or ATR3) were selected for ATR inhibition, one for ATM (ATM1) and an unrelated scramble sequence as a control. One day after the first transfection, cells were re-transfected with the same siRNA. Two days after
the second transfection, cells were exposed to 8Gy of IR, and harvested at 2 or 12 hours post-IR. Shown are images of western blots using ATR, ATM and phospho-p53ser18 antibodies. Tubulin antibody was used to check for equal loading. Images shown are representative data from three independent experiments.

**Fig.3 Inhibition of ATR/ATM kinase activity by PI-3 related kinase inhibitors.** Cells were treated for one hour with the indicated concentrations of (a) Wortmannin; or (b) ATM inhibitor KU55933 and then exposed to 8 Gy of IR. Cells were harvested at 2 and 12 hours post-IR, and p53ser18 phosphorylation status was analyzed by western blotting. Images shown are representative data from three independent experiments.

**Fig.4 ATM/Ku70 double deficient cells exhibit persistent p53ser18 phosphorylation.** (a) Two independent ATM/Ku70 double deficient cell lines were obtained after Cre-adenovirus treatment of the ATM-deficient, Ku homozygous conditional parent cell line, D14-3. A82-1 is an ATM-deficient cell line. Cells were exposed to 8 Gy of IR, harvested two hours after for western blotting using the indicated antibodies. (Refer to Materials and Methods Section and Supplementary Figure 2a for detailed strategy to generate these cells. (b) Cells were exposed to 8 Gy of IR, harvested at the indicated time points after irradiation and analyzed by western blotting. Alpha-tubulin was checked to confirm equal loading. Images shown are representative data from three independent experiments.

**Fig.5 ATR knockdown results in diminished p53ser18 phosphorylation in Ku/ATM double deficient cells.** (a) Two independent siRNA sequences (ATR1 or ATR3) were selected for ATR inhibition. A scramble sequence was used as a control. Transfections were conducted as previously described in Figure 2. (b) Cells were treated with the indicated concentrations of caffeine for one hour and exposed to 8 Gy of IR. Cells were harvested at 2 or 12 hours post-IR, and analyzed for p53ser18 phosphorylation by western blotting. Images shown are representative data from three independent experiments.

**Fig.6 A model for the involvement of Ku70/80 in the regulation of ATM and ATR activity during the DSB damage response.** Bar graphs show the relative contribution of each process to p53ser18 phosphorylation. (a) In the very early stages following IR of wild type cells, ATM directly phosphorylates p53ser18. (b) At 2 hours post-IR, direct ATM activity and ATM-dependent ATR activity mediate this phosphorylation. (c) At 12 hours post-IR, both ATM and ATR activities return to basal levels. (d) On the other hand, in Ku-deficient cells 2 hours post-IR, direct ATM activity and ATM-independent ATR activity phosphorylate p53ser18. (e) At 12 hours post-IR one or both processes may occur: (Possibility A) similar to that occurring at 2 hours post-IR and/or (Possibility B) direct ATM activity, ATM-dependent ATR activity and ATM-independent ATR activity phosphorylate p53ser18.

**SFig.1 Inhibition of ATR/ATM kinase activity using caffeine.** Cells were treated for one hour with the indicated concentrations of caffeine and then exposed to 8 Gy of IR. Cells were harvested at 2 and 12 hours post-IR, and p53ser18 phosphorylation status was analyzed by western blotting. Images shown are representative data from three independent experiments.

**SFig.2 ATM/Ku70 double deficient cell lines were obtained by Cre-recombinase treatment of ATM-deficient/Ku70-conditional cells.** (a) Strategy for selective targeting of Ku70 transcription start sites. The Ku70 locus, before and after exon2 disruption, is shown. LoxP sites are denoted as gray triangles. (b) ATM-deficient/Ku70-conditional cells were infected with Cre-adenovirus or LacZ-adenovirus. The cells were harvested at indicated time points and analyzed for Ku70 levels by western blotting using Ku70 (M-19), Ku70 (C-19) or tubulin. Ku70 expression gradually decreased and until it was no longer observable at 3 days post-infection. Ku70 levels exhibited no change upon separate infection with an unrelated LacZ adenovirus.
| Sample Type | Time (hr) | p53ser18 | Tubulin |
|-------------|-----------|----------|---------|
| PK34N (Wild type) | PC | | |
| | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK34N (Wild type) | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK33N (DNA-PKcs^/-) | PC | | |
| | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK33N (DNA-PKcs^/-) | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| STEFKu70 (Ku70^/-) | PC | | |
| | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| STEFKu70 (Ku70^/-) | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK/80-1A (Ku80^/- DNA-PKcs^+/^-) | PC | | |
| | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK/80-1A (Ku80^/- DNA-PKcs^+/^-) | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK/80-193A (Ku80^/- DNA-PKcs^-/-) | PC | | |
| | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
| PK/80-193A (Ku80^/- DNA-PKcs^-/-) | 0 | | |
| | 1 | | |
| | 2 | | |
| | 4 | | |
| | 8 | | |
| | 12 | | |
| | 16 | | |
Figure 2

|       | PC | ATR1 | ATR3 | ATM1 | Control | ATR1 | ATR3 | ATM1 | Control |
|-------|----|------|------|------|---------|------|------|------|---------|
| PK34N |     |      |      |      |         |      |      |      |         |
| ATM   |     |      |      |      |         |      |      |      |         |
| p53ser18 |      |      |      |      |         |      |      |      |         |
| tubulin |     |      |      |      |         |      |      |      |         |
| STEFKu70 |    |      |      |      |         |      |      |      |         |
| ATM   |     |      |      |      |         |      |      |      |         |
| p53ser18 |      |      |      |      |         |      |      |      |         |
| tubulin |     |      |      |      |         |      |      |      |         |
| PK/80-1A |   |      |      |      |         |      |      |      |         |
| ATM   |     |      |      |      |         |      |      |      |         |
| p53ser18 |      |      |      |      |         |      |      |      |         |
| tubulin |     |      |      |      |         |      |      |      |         |
| PK/80-193A |  |      |      |      |         |      |      |      |         |
| ATM   |     |      |      |      |         |      |      |      |         |
| p53ser18 |      |      |      |      |         |      |      |      |         |
| tubulin |     |      |      |      |         |      |      |      |         |
Figure 3

a. Wortmannin

|         | 2hr | 12hr |
|---------|-----|------|
| PK34N   |     |      |
| STEFKu70|     |      |
| PK/80-1A|     |      |
| PK/80-193A|   |      |

b. KU55933

|         | 2hr | 12hr |
|---------|-----|------|
| PK34N   |     |      |
| STEFKu70|     |      |
| PK/80-1A|     |      |
| PK/80-193A|   |      |
Figure 4

a.

|               | PK34N | STEF    | Ku70 (M-19) | ATM | Tubulin | p53ser18 |
|---------------|-------|---------|-------------|-----|---------|----------|
| IR           | -     | +       | -           | -   | +       | -        |
| A82-1        | -     | +       | -           | -   | +       | -        |
| D14-3        | -     | +       | -           | -   | +       | -        |
| FX1          | -     | +       | -           | -   | +       | -        |
| FX2          | -     | +       | -           | -   | +       | -        |

b.

|               | PK34N | D14-3  | D14-3       | D14-3     | D14-3 Fx1 | D14-3 Fx2 |
|---------------|-------|--------|-------------|-----------|------------|------------|
| PC           | 0     | 1      | 2           | 4         | 8          | 12         |
|              | 16    |        |             |           |            |            |
| p53ser18     |       |        |             |           |            |            |
| tubulin      |       |        |             |           |            |            |

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Figure 5

a. 

|            | 2hr          | 12hr         | siRNA  |
|------------|--------------|--------------|--------|
|            | ATR1  ATR3 Cont | ATR1  ATR3 Cont |        |
| D14-3      |              |              |        |
| Fx1        |              |              |        |
| FX2        |              |              |        |
| P53ser18   |              |              |        |
| tubulin    |              |              |        |

b. 

|            | 2hr          | 12hr         |
|------------|--------------|--------------|
|            | PC 0 1 3 10 | 0 1 3 10 (mM)|
| PK34N      |              |              |
| D14-3      |              |              |
| Fx1        |              |              |
| FX2        |              |              |
| P53ser18   |              |              |
| tubulin    |              |              |
Wild type

(a) IR 30min

(b) 2hrs

(c) 12hrs

Ku70/80-deficient

(d) 2hrs

(e) 12hrs (Possibility A) 12hrs (Possibility B)

Figure 6
Ku70/80 modulates ATM and ATR signaling pathways in response to DNA double-strand breaks
Nozomi Tomimatsu, Candice G. T. Tahimic, Akihiro Otsuki, Sandeep Burma, Akiko Fukuhara, Kenzo Sato, Goshi Shiota, Mitsuo Oshimura, David J. Chen and Akihiro Kurimasa

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