Recruitment and activation of the ATM kinase in the absence of DNA-damage sensors

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Two kinases, ATM and DNA-PKcs, control rapid responses to DNA double-strand breaks (DSBs). The paradigm for ATM control is recruitment and activation by the Mre11–Rad50–NBS1 (MRN) sensor complex, whereas DNA-PKcs requires the sensor Ku (Ku70–Ku80). Using mouse cells containing targeted mutant alleles of Mre11 (Mre11a) and/or Ku70 (Xrcc6), together with pharmacologic kinase inhibition, we demonstrate that ATM can be activated by DSBs in the absence of MRN. When MRN is deficient, DNA-PKcs efficiently substitutes for ATM in facilitating local chromatin responses. In the absence of both MRN and Ku, ATM is recruited to chromatin, where it phosphorylates H2AX and triggers the G2-M cell-cycle checkpoint, but the DNA-repair functions of MRN are not restored. These results suggest that, in contrast to straightforward recruitment and activation by MRN, a complex interplay between sensors has a substantial role in ATM control.

DNA DSBs are a highly toxic form of DNA damage and a potent cause of genome instability. In eukaryotes, two major pathways exist to repair DSBs: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). Before actual repair, systems must be in place to detect DNA lesions and to initiate signaling cascades that alter many aspects of cellular physiology. Collectively these DNA-damage responses comprise an intricate network of proteins in a complex choreography of events. Failure to correctly repair DSBs can lead to carcinogenesis as well as neurological and developmental disorders. The Ku complex also binds DSBs, but it specifically activates the DNA-PKcs kinase; together, Ku and DNA-PKcs make up the DNA-PK holoenzyme. Ku is one of the most abundant cellular proteins, and it exhibits high affinity for double-stranded DNA ends. Unlike the many functions of ATM, the primary role of DNA-PK is direct participation in DSB repair via NHEJ. In this role DNA-PKcs itself is the most well-characterized substrate of the DNA-PK holoenzyme. Mice lacking DNA-PKcs, and those lacking Ku70 or Ku80, are immunodeficient, owing to defective V(D)J recombination, which requires NHEJ.

It is clear that the cellular response to DSBs is a sophisticated web of operations involving many proteins that must be tightly regulated in order to generate the necessary outcome. A deeper understanding of these processes is key to decoding the pathogenesis of cancer and designing successful treatments for this disease. However, unraveling this web is a difficult challenge. To gain mechanistic understanding of the earliest events in the mammalian DSB response, we examined cells lacking one or both of the DSB-sensor complexes. We discovered that removal of Ku in the absence of the MRN complex restores several ATM-dependent responses, thus demonstrating that MRN is not absolutely required for recruitment and activation of ATM.

RESULTS

Generation of MRN-deficient and MRN- and Ku-deficient cells

We disabled the MRN complex through use of mouse embryonic fibroblasts (MEFs) that originally contained one Mre11 null allele.
(Mre11−/−) and one Cre/LoxP conditional allele (Mre11cond/+) (Fig. 1b). Exposure to Cre recombinase via adenovirus converted these cells to Mre11−/+, which also causes deficiency of Rad50 and NBS1 (termed MRN deficiency) (Fig. 1c and Supplementary Fig. 1), presumably because of instability of the proteins in the absence of Mre11 (ref. 14). Control cells used for experiments were initially Mre11cond/++, which we converted to Mre11−/− via Cre recombinase in parallel with other genotypes to control for effects of viral delivery of Cre and for LoxP recombination at a target locus. Through mouse breeding, we also generated MEFs of these MRN genotypes combined with conditional allele (bottom) 14. Red outline, histidine in exon 5 (gray box) essential for nuclease activities; lines, introns; triangles, LoxP sites; asterisk, H129N mutation. (Fig. 1).

Rapid recruitment of MRN and Ku to DNA damage
To gain understanding of the interplay between sensor complexes in the immediate aftermath of DSB induction, we used live-cell imaging to examine localization of MRN and Ku to sites of DNA damage. We used GFP-tagged NBS1 to track MRN recruitment to laser-induced damage in real time. MRN was recruited within 15 to 30 s after damage, and Ku deficiency did not appear to affect its recruitment (Fig. 2a). To track Ku, we introduced GFP-tagged Ku70 and Ku80 and found that Ku recruitment was faster than laser stripes could be generated. Instead, we pulsed the laser at a single location while simultaneously recording video instead of time-lapse images. We detected Ku at damage sites within 1s after damage, and it continued to accumulate for approximately 10 s in most cells (Fig. 2b, Supplementary Fig. 2 and Supplementary Movie 1). Loss of the MRN complex did not appear to affect Ku recruitment. Therefore, immediately after DNA damage, the absence of either sensor does not substantially affect overall accumulation of the other.

Restoration of G2-M checkpoint in MRN- and Ku-deficient cells
The ATM kinase controls a crucial DNA-damage checkpoint at the transition between the G2 and M phases of the cell cycle11. This checkpoint prevents the passage of broken chromosomes to daughter cells, and its absence contributes to the cancer predisposition observed in patients with AT or certain inherited mutations in MRN genes13. We analyzed the G2-M checkpoint by assessing the effect of ionizing radiation (IR) on the mitotic index, as measured by the percentage of cells positive for the mitosis-entry marker phosphorylated (phospho-) histone H3S10. An intact checkpoint is reflected by a substantial reduction in the percentage of mitotic cells after IR (reduced mitotic index), whereas a defective checkpoint manifests as a comparatively higher percentage of cells passing into M phase (higher mitotic index).

As anticipated, we observed higher relative percentages of Mre11−−/− (ref. 22) and ATM−−/− (ref. 23) cells in M phase after IR (Fig. 3a). Unexpectedly, cells lacking both Mre11 and Ku70 displayed an intact G2-M checkpoint. Given the absence of both sensors, this suggested that another kinase (other than DNA-PKcs) may be substituting for ATM or that the G2-M checkpoint does not require a kinase. Strikingly, when we performed these experiments in the presence of pharmacologic inhibitors of ATM or DNA-PKcs, we found that the G2-M checkpoint was dependent on ATM (Fig. 3b). Thus, the requirement for MRN in the ATM-dependent G2-M checkpoint appears to be alleviated by the absence of Ku.

MRN DNA-repair functions are not rescued by Ku deficiency
In addition to initiating signaling, MRN and Ku function directly in the repair of DSBs. The bypass of MRN’s requirement in activating the ATM-dependent G2-M checkpoint raises the possibility that requirements for MRN during DSB repair may be alleviated through removal of Ku. This notion is supported by studies in budding or fission yeast demonstrating that the IR-induced lethality of Mre11 deficiency is suppressed by deletion of Ku24,25. We therefore examined the effects of Ku deficiency on MRN-dependent repair functions by examining IR sensitivities and the efficiencies of HDR in cells lacking one or both sensors. From the IR sensitivity of Mre11−−/− Ku70−−/− cells, we found no evidence for rescue of IR hypersensitivity by removal of Ku (Fig. 4a).

We used an integrated direct repeat (DR)-GFP reporter assay to measure HDR26 (Fig. 4b). The Mre11−−/− genotype indicates the parental cell line with two distinct Mre11 alleles (conditional (c) and null (−)). To provide matched controls for Mre11 deletion, we treated cells with either Adeno-Cre recombinase to generate Mre11−−/− or an empty adenovirus to maintain the Mre11−−/− genotype. Mre11 deficiency decreased HDR, whereas Ku deficiency elevated HDR relative to that of control cells (Fig. 4c), as has been reported previously14,26. In Ku-deficient cells, further removal of Mre11 decreased HDR by a percentage comparable to that caused by Mre11 removal in control cells (Fig. 4d). Therefore we find no evidence for rescue of MRN-dependent HDR by Ku deficiency (Fig. 4c,d). This is consistent with our finding that...
Ku70 deficiency provided no rescue of Mre11−/− early mouse embryonic lethality (Supplementary Table 1), because functional HDR is required for early embryonic development27. Together, these findings indicate that in mammals, removal of Ku can largely bypass the need for MRN to activate the ATM-dependent G2-M checkpoint but not the need for MRN to facilitate homology-directed DSB repair.

ATM recruitment and activation in the absence of MRN and Ku

ATM is rapidly recruited to chromatin in the vicinity of DSBs28. This recruitment has been presumed to require interaction with MRN. We therefore used cellular fractionation to assess whether ATM is recruited to chromatin in the absence of MRN and Ku. We observed IR-induced chromatin localization of ATM in control cells (Mre11+/− Ku70+/+) but not in MRN-deficient cells (Mre11−/− Ku70+/+) (Fig. 5a and Supplementary Data Set 1). Strikingly, when Ku was also absent (Mre11−/− Ku70−/−) ATM chromatin localization was restored. Thus, the absence of Ku reveals that MRN is not strictly required for DNA damage–induced ATM chromatin localization.

To further understand ATM recruitment and the intact ATM-dependent checkpoint in the absence of DSB sensors, we examined one of the earliest signaling events in the DSB response, phosphorylation of histone H2AX. This specialized histone is present in 2% to 25% of nucleosomes, depending on cell type, and is phosphorylated (with the phosphorylated form denoted γH2AX) for megabase regions on either side of a DSB29. γH2AX then serves as a platform for more extensive chromatin changes that are required to propagate DNA-damage responses11 (schematic in Fig. 1a). We induced DSBs by exposing MEFs to IR and measured total cellular γH2AX formation by immunoblotting. In addition, we determined the relative contributions of ATM and DNA-PKcs to this signaling by pretreating cells with specific pharmacologic kinase inhibitors.

In control cells (Mre11+/+), the ATM inhibitor markedly decreased H2AX phosphorylation, whereas DNA-PKcs inhibition had little effect (Fig. 5b and Supplementary Data Set 1). In contrast, we observed the opposite pattern in MRN-deficient cells (Mre11−/−), wherein the DNA-PKcs inhibitor reduced H2AX phosphorylation to near-background levels. This result is consistent with models depicting a requirement for MRN in ATM recruitment and activation9–11 (schematic in Fig. 1a), and it demonstrates that DNA-PKcs can very efficiently substitute for ATM in phosphorylating H2AX.

Figure 2 MRN and Ku relocate to DSBs independently in the early DSB response. (a, b) Live-cell imaging of MRN (a) and Ku (b) relocation to laser microirradiation–induced damage. Two representative cells are shown per genotype, from a total of 45 and 88 cells per genotype in a and b, respectively; the experiments were performed with 3 cell-culture replicates. Scale bars, 10 μm. (a) GFP-tagged NBS1, monitored in live cells by time-lapse photography during fluorescence microscopy. (b) GFP-tagged Ku70 and Ku80, monitored in live cells via fluorescence microscopy video capture. Individual frames corresponding to indicated time points (left) are shown. Arrowheads indicate spot of laser target.

Figure 3 Ku loss bypasses the need for MRN in activating the G2-M checkpoint. (a) Assessment of the G2-M checkpoint by comparison of mitotic indices (percentage phospho-H3S10–positive cells) before and 1 h after 10 Gy IR. Mitotic index, +IR/–IR ratio. Shorter bars reflect intact checkpoint function. Data shown are means and s.e.m. (n = 3 cell-culture replicates). (b) Assessment of the G2-M checkpoint in untreated cells or cells pretreated with kinase inhibitors (ATMi, ATM inhibitor; PKi, DNA-PK inhibitor), as in a. Data shown are means and s.e.m. (n = 3 cell-culture replicates).
focus formation. We observed that, in agreement with immunoblot data (Fig. 5b), γH2AX foci formation shifted dependency from ATM to DNA-PKcs when MRN was absent (Fig. 5c and Supplementary Fig. 4). Importantly, when Ku was also removed, dependency returned to ATM. In each genotype, the majority of cells (65 to 80%) contained ten or more γH2AX foci, and the morphologies of these foci were all similar (Fig. 5c and Supplementary Fig. 4). Thus, as in normal cells, ATM activated in the absence of MRN and Ku is recruited to chromatin in the vicinity of DSBs to phosphorylate H2AX.

Rapid MDC1 recruitment in the absence of MRN and Ku

After H2AX is phosphorylated, it is bound by mediator of DNA damage checkpoint 1 (MDC1)\(^{30,31}\). MDC1 then promotes recruitment of DNA-damage-response and checkpoint proteins to the chromatin-flanking DSBs, thus ultimately leading to initiation of the G2-M cell-cycle checkpoint\(^{31–33}\). To gain further insight into the newly
discovered MRN-independent ATM activation and G2-M checkpoint initiation, we examined GFP-tagged MDC1 recruitment with live-cell imaging after laser microirradiation. This approach permits visualization of recruitment within seconds to minutes after damage, far faster than timescales permitted in immunofluorescence-based experiments. Thus, ATM activation can be compared within the earliest timeframe of the DNA-damage response.

We observed MDC1 recruitment by 15 s, and this recruitment was dependent on ATM (Fig. 6), as previously shown. When MRN was rendered deficient, MDC1 accumulated as rapidly and intensely as in control cells. However, this recruitment was dependent on DNA-PKcs (Fig. 6). Thus, even during the rapid events immediately following damage, DNA-PKcs can locally substitute for ATM when MRN is absent. Further removal of Ku (MRN and Ku deficiency) had little effect on MDC1 recruitment, causing only a subtle delay of less than 15 s; however, strikingly, this robust recruitment was ATM dependent (Fig. 6). Therefore, even within the first minute of the DSB response, ATM can function in the absence of MRN and Ku.

ATM activation over broad experimental conditions

We further investigated MRN-independent ATM activation by comparing various parameters to ATM activation in control cells. The pattern of γH2AX kinase dependency, determined over a variety of IR doses ranging from 0.5 to 75 Gy, did not differ at any dose tested (Supplementary Fig. 5a–c). We also compared γH2AX levels over a time course after IR ranging from 15 min to 10 h (Fig. 7a and Supplementary Fig. 5d). H2AX phosphorylation was readily observable by immunoblotting in MRN and Ku deficiency throughout the time course. The peak intensity for both genotypes was similar, though it appeared delayed in MRN- and Ku-deficient cells: control cells achieved peak signal intensity by 30 min after IR, whereas Mre11+/–Ku70−/− cells did so 2 h after IR (Fig. 7a). In addition, this time course revealed a persistence of H2AX phosphorylation in MRN- and Ku-deficient cells compared to controls. This result is consistent with the presence of unrepaired DSBs and is not unexpected given the HDR defect caused by Mre11 deficiency (Fig. 4c,d) and the NHEJ defect caused by Ku70 deficiency.

End resection and kinase activation

In the presence of MRN, single-stranded DNA (ssDNA) at a break has been shown in vitro to be a potent activator of ATM. Recent work has demonstrated the importance of Mre11 nuclease–dependent end resection in the initiation and choice of DNA-repair pathways; however, its role in kinase activation is less understood. To investigate roles of Mre11 nuclease activity, we generated Ku-null MEFs expressing Mre11 with a targeted single amino acid change (Mre11H129N) that abrogates DNA nuclease activities while maintaining normal MRN interactions and structure (schematic in Fig. 1b). We found that Mre11 nuclease deficiency maintained ATM as the primary kinase after IR, with or without Ku70 (Fig. 7b and Supplementary Fig. 6). This demonstrates that H2AX can be efficiently phosphorylated by DNA-PKcs when MRN is absent but not when a nuclease-deficient version of the complex is present. Therefore MRN-dependent resection appears to be dispensable in the rapid signaling response to DSBs.

The ATR kinase, another PIKK family member that functions in DNA-damage responses, is recruited and activated by ssDNA. It has been shown that ATR can phosphorylate and activate ATM. Although Mre11 nuclease activity is dispensable for rapid signaling after damage, it remains possible that other nucleases could generate the ssDNA necessary to activate ATR. To investigate a role for ATR in MRN-independent ATM activation, we used a specific ATR kinase inhibitor to examine DNA-repair signaling in cells lacking MRN and Ku. Chk1 at S345 is an established target of the ATR kinase, and its phosphorylation is used as a readout of ATR activity and inhibition. We found that ATM activation by IR in...
MRN- and Ku-deficient cells was similar to that in control cells and did not depend on ATR (Fig. 7c).

Our results have shown that in the absence of MRN, DNA-PKcs can very efficiently substitute for ATM in phosphorylating H2AX during the DSB response (Figs. 5b and 7d–f and Supplementary Figs. 3a and 7a,e). However, when MRN is present but dysfunctional (nucleasie deficient), DNA-PKcs has no detectable effect on H2AX phosphorylation (Fig. 7b,d and Supplementary Fig. 7c,e). This suggests that the presence of MRN at DSBs somehow prohibits DNA-PKcs from having a major role in H2AX phosphorylation. To assess this in more detail, we examined H2AX phosphorylation in the presence of the MRN complex but the absence of the ATM kinase, conditions in which another kinase would be needed to phosphorylate H2AX in response to damage. We found that DNA-PKcs has a limited ability to phosphorylate H2AX in the absence of ATM, but not to the extent observed in MRN-deficient cells (Fig. 7d,e and Supplementary Fig. 7d,e). These data support the hypothesis that the presence of the MRN complex at DSBs hinders the ability of DNA-PKcs to phosphorylate H2AX.

In normal cells that have suffered DSBs, active ATM is autophosphorylated at S1981 (S1987 in mice). Although this autophosphorylation site is not required for ATM kinase activity, it nonetheless has served as a convenient experimental marker for ATM activation. Interestingly, we observed that ATM lacks S1981 phosphorylation in MRN- and Ku-deficient cells (Fig. 7f and Supplementary Figs. 5e and 7a,b). Therefore, the phosphorylation of ATM S1981 provides a molecular distinction between ATM activated in the presence versus the absence of MRN and Ku.

**DISCUSSION**

Here we have shown that in the absence of MRN, removal of Ku restores ATM chromatin localization, kinase activation and the G2-M cell-cycle checkpoint. In contrast, DNA-repair functions of MRN are not restored in the absence of Ku. These findings reveal that MRN is not absolutely required for recruitment and allosteric activation of ATM but that it instead functions to maximize or sustain ATM-dependent DSB responses. Unlike simple recruitment, activation of ATM appears to be strongly influenced by a rapid interplay between MRN and Ku occurring within seconds after DNA damage.

Previous studies have shown that Ku and MRN can compete for binding to DSBs in vitro, and Ku must be removed from DNA ends to allow for HDR. Together, these results suggest some amount of competition between MRN and Ku in vivo. However, findings from our live cell–imaging studies suggest that simple competition does not predominate within the earliest seconds of the DSB response (Fig. 2, Supplementary Fig. 2 and Supplementary Movie 1). These studies support the notion that both sensors bind DSBs rapidly, with Ku binding first and MRN joining shortly thereafter. Neither complex appears to require the other for recruitment to DSBs, and, importantly, within this narrow time frame that includes kinase activation, the absence of one sensor does not measurably affect the recruitment of the other. These findings substantially constrain the mechanism at work in the early DSB response. In this context, we speculate that there is a level of opposition in the functions of MRN and Ku at DNA ends that does not involve simple competition for initial binding.

In principle, the restoration of ATM function in MRN and Ku deficient cells is similar to that in control cells and did not depend on ATR (Fig. 7c).

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Ku-deficient cells without the need for Ku deficiency. However, we did not observe this in our examinations of ATM-substrate phosphorylation and γH2AX kinetics (Figs. 5b and 7c–f and Supplementary Figs. 3a, 5b,c and 7a,e). Similarly, our observation that DNA-PKcs was able to substitute for ATM in phosphorylating H2AX when MRN was absent (Fig. 5b) raised the possibility that increased DNA-PKcs activity could result from a lack of ATM activity rather than from the absence of MRN. However, treatment with the ATM inhibitor in control cells did not allow DNA-PKcs to substitute for ATM (Figs. 5b and 7c–f and Supplementary Figs. 3a, 5b,c and 7a). We tested this hypothesis further by examining ATM-knockout cells in the presence and absence of the DNA-PKcs inhibitor and then comparing γH2AX immunoblot signals (Fig. 7d,e and Supplementary Fig. 7d,e). In the absence of ATM, DNA-PKcs did show a modest ability to phosphorylate H2AX, but levels clearly remained below those observed in MRN-deficient cells, which were indistinguishable from control cells. These findings suggest that interplay among the DSB sensors plays the major part in controlling ATM activation, and kinase cross-talk makes a more minor contribution.

We uncovered several lines of evidence that support the notion that MRN, although it is not essential for initial ATM activation, maximizes and sustains ATM-dependent responses. Phosphorylation of ATM substrates Kap1 and SMC1 in cells lacking MRN and Ku was not as robust as in control cells (Supplementary Fig. 3a,b and 7d). In both cases, IR-induced phosphorylation was fully ATM dependent but was reduced by 60% to 70% (Supplementary Fig. 3b). Detailed examination of the time course of H2AX phosphorylation revealed that peak levels of γH2AX were similar but were delayed approximately 1.5 h in MRN- and Ku-deficient cells compared to controls (Fig. 7a). Live-cell imaging of γH2AX-dependent MDC1 recruitment demonstrated that MDC1 was rapidly recruited to sites of damage in cells of all the genotypes examined, but this recruitment was slightly decreased in MRN- and Ku-deficient cells 15 s after damage (Fig. 6). This level of recruitment is near the limit of detection of the instrument used for this study, and thus interpretations must be restrained. Therefore, although antibody-based approaches that measure responses over longer times suggest a more substantial delay, our live-cell imaging indicated that any delay in ATM-dependent signaling in the absence of MRN and Ku is minimal during the critical first minute after DNA damage.

For MRN to influence ATM-dependent DSB responses, it must overcome the more rapid binding by Ku in vivo, which is capable of protecting ends42–44. The precise nature of how MRN overcomes this protection will require future studies, but the complex could conceivably eject Ku by converting DSBs to ssDNA ends, which are a poor substrate for Ku binding45,46. MRN is known to possess two activities that can generate ssDNA: the nuclease activities of Mre11 and ATP-dependent DNA unwinding by Rad50 (ref. 46). The endonuclease activity of Mre11 initiates processing at a short distance away from the break and could therefore occur even when Ku is bound45. However, we have ruled out a requirement for Mre11 nuclease activity in kinase activation (Fig. 7b,d,e and Supplementary Figs. 6 and 7c,e), thus suggesting a role for Rad50’s ATP-dependent DNA unwinding and the recently discovered dramatic conformational changes in Rad50 structure42,47.

The newly uncovered MRN-independent activation of ATM presented here represents a notable advance in understanding of the early DSB response. The ability of ATM to function in the absence of MRN helps to explain studies in mice engineered to lack the ATM-interacting domain of NBS1, which display surprisingly minimal AT-like phenotypes48,49. The strong requirement for MRN only in the presence of Ku revealed in this study suggests that a key function for MRN in ATM activation is to oppose Ku at DSBs to expose DNA ends. This notion is supported by prior in vitro studies12,15,50, including a study from Xenopus oocyte extracts that has shown that ATM can be activated in the absence of MRN, but only when relatively higher levels of DNA ends are added15. Further studies will be needed to determine whether an unknown factor is required for ATM recruitment and activation or whether ATM is in fact activated directly by DNA ends in vivo50.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.H. planned and performed most experiments, analyzed and interpreted the data and participated in writing all portions of the manuscript. Y.W. performed and interpreted experiments in Figure 7d,e and Supplementary Figures 6 and 7e. M.J.M. performed and interpreted immunofluorescent foci experiments in Figure 5c and Supplementary Figures 3c,d and 4, and assisted A.J.H. with the laser microirradiation experiments in Figures 2a and 6. J.B. performed initial mouse breeding and analyses of Mendelian inheritance (Supplementary Table 1). D.O.F. participated in the design of all experiments as well as analyses and interpretation of data, and in writing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
32. Lou, Z.

29. Rogakou, E.P., Boon, C., Redon, C. & Bonner, W.M. Megabase chromatin domains

28. Andegeko, Y.

27. Helleday, T. Pathways for mitotic homologous recombination in mammalian cells.

26. Pierce, A.J., Johnson, R.D., Thompson, L.H. & Jasin, M. XRCC3 promotes homology-

25. Tomita, K.

24. Bressan, D.A., Baxter, B.K. & Petrini, J.H. The Mre11-Rad50-Xrs2 protein complex

23. Shiloh, Y. ATM and related protein kinases: safeguarding genome integrity.

22. Carson, C.T.

21. Gu, Y.

20. Jhappan, C., Morse, H.C. III., Fleischmann, R.D., Gottesman, M.M. & Merlino, G.

19. Gao, Y.

18. Davis, A.J., Chen, B.P. & Chen, D.J. DNA-PK: a dynamic enzyme in a versatile DSB

17. DNA-PKcs: a T-cell tumour suppressor encoded at the mouse scid locus.

16. Dynan, W.S. & Yoo, S. Interaction of Ku protein and DNA-dependent protein kinase

15. Dupré, A., Boyer-Chatenet, L. & Gautier, J. Two-step activation of ATM by DNA and

the Mre11-Rad50-Nbs1 complex. Nat. Struct. Mol. Biol. 13, 451–457 (2006).

14. Dyman, W.S. & Yoo, S. Interaction of Ku protein and DNA-dependent protein kinase

catalytic subunit with nucleic acids. Nucleic Acids Res. 26, 1551–1559 (1998).

13. Walker, J.R., Corpina, R.A. & Goldberg, J. Structure of the Ku heterodimer bound to

DNA and its implications for double-strand break repair. Nature 412, 607–614 (2001).

12. Davis, A.J., Chen, B.P. & Chen, D.J. DNA-PK: a dynamic enzyme in a versatile DSB

repair pathway. DNA Repair (Amst.) 17, 21–29 (2014).

11. Gao, Y. et al. A targeted DNA-PKcs-null mutation reveals DNA-PK-independent

functions for Ku in V(D)J recombination. Immunity 9, 367–376 (1998).

10. Jaqpan, G., Morse, H.C. III., Fleischmann, R.D., Gottesman, M.M. & Merlino, G.

DNA-PKcs: a T-cell tumour suppressor encoded at the mouse scid locus. Nat. Genet.

17, 483–486 (1997).

9. Gu, Y. et al. Growth retardation and leaky SCID phenotype of Ku70-deficient mice. Immunity 7, 653–665 (1997).

8. Carson, C.T. et al. The Mre11 complex is required for ATM activation and the

G2/M checkpoint. EMBO J. 22, 6610–6620 (2003).

7. Shiloh, Y. ATM and related protein kinases: safeguarding genome integrity. Nat.

Rev. Cancer 3, 155–168 (2003).

6. Bressan, D.A., Baxter, B.K. & Petrini, J.H. The Mre11-Rad50-Xrs2 protein complex

facilitates homologous recombination-based double-strand break repair in

Saccharomyces cerevisiae. Mol. Cell. Biol. 19, 7681–7687 (1999).

5. Tomita, K. et al. Competition between the Rad50 complex and the Ku heterodimer

reveals a role for Exo1 in processing double-strand breaks but not telomeres. Mol. Cell.

23, 5186–5197 (2003).

4. Pierie, A.J., Johnson, R.D., Thompson, L.H. & Jasmin, M. XRCC3 promotes homology-

directed repair of DNA damage in mammalian cells. Genes Dev. 13, 2633–2638

(1999).

3. Helbeday, T. Pathways for mitotic homologous recombination in mammalian cells.

Mutat. Res. 532, 103–115 (2003).

2. Andegeko, Y. et al. Nuclear retention of ATM at sites of DNA double strand breaks.

J. Biol. Chem. 276, 38224–38230 (2001).

1. Rogakou, E.P., Boon, C., Redon, C. & Bonner, W.M. Megabase chromatin domains

involved in DNA double-strand breaks in vivo. J. Cell Biol. 146, 905–916 (1999).

30. Xu, X. & Stern, D.F. NFB1/KIAA0170 is a chromatin-associated protein involved in

DNA damage signaling pathways. J. Biol. Chem. 278, 8795–8803 (2003).

29. Stucki, M. et al. MDC1 directly binds phosphorylated histone H2AX to regulate

cellular responses to DNA double-strand breaks. Cell 123, 1213–1226 (2005).

28. Lou, Z. et al. MDC1 maintains genomic stability by participating in the amplification of

ATM-dependent DNA damage signals. Mol. Cell 21, 187–200 (2006).

27. Lan, L. et al. Accumulation of Werner protein at DNA double-strand breaks in

human cells. J. Cell Sci. 118, 4153–4162 (2005).

26. Shibata, A. et al. DNA double-strand break repair pathway choice is directed by

distinct MRE11 nuclelease activities. Mol. Cell 53, 7–18 (2014).

25. Williams, R.S. et al. Mre11 dimers coordinate DNA end bridging and nuclease

processing in double-strand-break repair. Cell 135, 97–109 (2008).

24. Zou, L. & Elledge, S.J. Sensing DNA damage through ATR/PRC recognition of RPA-

ssDNA complexes. Science 300, 1542–1548 (2003).

23. Shao, Z.

22. Blier, P.R., Griffith, A.J., Craft, J. & Hardin, J.A. Binding of Ku protein to

the 3′ end of ssDNA complexes. J. Biol. Chem. 273, 27800–27806 (1998).

21. Tomita, K. et al. Two-step activation of ATM by DNA double-strand breaks.

EMBO J. 17, 3744–3752 (1998).

20. Prevo, R. et al. The novel ATR inhibitor VE-821 increases sensitivity of pancreatic

cancer cells to radiation and chemotherapy. Cancer Biol. Ther. 13, 1072–1081

(2012).

19. Shibata, A. et al. DNA double-strand break repair pathway choice is directed by

distinct MRE11 nuclelease activities. Mol. Cell 53, 7–18 (2014).

18. Williams, R.S. et al. Mre11 dimers coordinate DNA end bridging and nuclease

processing in double-strand-break repair. Cell 135, 97–109 (2008).

17. Zou, L. & Elledge, S.J. Sensing DNA damage through ATR/PRC recognition of RPA-

ssDNA complexes. Science 300, 1542–1548 (2003).

16. Shao, Z. et al. Persistently bound Ku at DNA ends attenuates DNA end resection

and homologous recombination. DNA Repair (Amst.) 11, 310–316 (2012).

15. Blier, P.R., Griffith, A.J., Craft, J. & Hardin, J.A. Binding of Ku protein to

DNA: measurement of affinity for ends and demonstration of binding to nicks. J.

Biol. Chem. 268, 7594–7601 (1993).

14. Pauli, T.T. & Deshpande, R.A. The Mre11/Rad50/Nbs1 complex: recent insights into

catalytic activities and ATP-driven conformational changes. Exp. Cell Res. 329,

139–147 (2014).

13. Shaw, A. et al. ATP-driven Rad50 conformations regulate DNA tethering, end

resection, and ATM checkpoint signaling. EMBO J. 33, 482–500 (2014).

12. Difilippantonio, S. et al. Distinct domains in Nbs1 regulate irradiation-induced

checkpoints and apoptosis. J. Exp. Med. 204, 1003–1011 (2007).

11. Stracker, T.H., Morales, M., Couto, S.S., Hussein, H. & Petrini, J.H. The carboxy

terminus of NBS1 is required for induction of apoptosis by the MRE11 complex. Nature

447, 218–221 (2007).

10. Smith, G.C. et al. Purification and DNA binding properties of the ataxia-telangiectasia
geneproduct ATM. Proc. Natl. Acad. Sci. USA 96, 11134–11139 (1999).
ONLINE METHODS

Growth and analysis of MEFs. All MEFs used in this study were derived in house from day E13.5 embryos and grown in standard culture conditions as previously described55. MEFs tested negative for mycoplasma contamination, and genotypes were confirmed by PCR22 and western blotting (Fig. 1c and Supplementary Fig. 1). Primary MEFs were immortalized by transfection with pBsSVD2005 (SV40 large T antigen expression vector). Adeno-Cre at an MOI of 500 was used. MEFs were grown for 3 d after infection and split once before plating for experiments. Inhibitors for the following kinases were used as follows: ATM, KU55933 (refs. 51,52) (10 μM or 20 μM for 1–2 h, as indicated, Tocris Biosciences); DNA-PK, NU7026 (refs. 53,54) (20 μM for 2 h, Tocris Biosciences); ATR, VE-821 (ref. 39) (10 μM for 2 h, Axon Medchem).

Rescue of embryonic lethality. Mice were of C57B6/129sv mixed background, with an equal mix of males and females. χ² analysis was used to determine the significance of any observed differences between the actual and expected results of the cross performed. No blinding, randomization or power calculation for sample size was performed. The University of Michigan’s University Committee on the Use and Care of Animals (UCUCA) approved all mouse procedures carried out in this study in accordance with an approved protocol.

Western blotting. Cell extracts were prepared in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% C24H39O4Na, 0.1% SDS, and 50 mM Tris-Cl, pH 8.0) or Laemmli buffer (4% SDS, 20% glycerol, and 120 mM Tris-HCl, pH 6.8), resolved by SDS-PAGE and transferred with standard procedures. Primary antibodies specific for the following proteins were used: Mre11 (Cell Signaling, 4895); Rad50 (Bethyl Laboratories, A300-184A)55; NBS1 (Novus Biologicals, NB10-57272); γH2AX and H2AX (EMD Millipore, 05-636 and 07-627)56; pChk1345 (Cell Signaling, 2348); Kap1 (Cell Signaling, 4124); pKap1S824 (Bethyl Laboratories, A300-767A); pSMC1S957 (Cell Signaling, 4805); ATM (Cell Signaling, 2873); pATM1980 (Rockland Immunochemicals, 200-301-400)55; Ku70 (Cell Signaling, 4588); Ku86 (Santa Cruz Biotechnology, sc9034); DNA-PKcs (Kamiya Biomedical, MC-365); Topol (BD Biosciences, 556597); and GAPDH (Cell Signaling, 2118). Validation of all primary antibodies unless otherwise referenced was provided by the manufacturer. Secondary antibodies for western blots wereIRDy-conjugated goat anti-rabbit or anti-mouse (Li-Cor Biosciences, 925-32210, 925-32211, 925-68070, 925-68071). Original uncropped images of key western blots in this study can be found in Supplementary Data Set 1.

Quantification of band intensities was performed with Li-Cor Odyssey 2.1 software. For each experiment, the level of the appropriate loading control in the unirradiated sample was set to one, and any differences in loading among the other samples were determined relative to this sample (‘normalized’). Phosphoprotein levels were then corrected to account for the normalized loading-control level for each sample (i.e., γH2AX level divided by normalized GAPDH).

Biochemical fractionation. Fractionation experiments were carried out as previously described24. Treated or mock-treated cells were washed with ice-cold PBS, and cell fractionation was carried out by consecutive extractions with increasing detergent concentration. Cell pellets were first resuspended for 5 min on ice in fractionation buffer I (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% IGEPAL CA-630) supplemented with protease and phosphatase inhibitors. After centrifugation at 1,000 g, the supernatant is fraction II, nuclear. The pellets were finally lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% C24H39O4Na, 0.1% SDS, and 50 mM Tris-Cl, pH 8.0) or Laemmli buffer (4% SDS, 20% glycerol, and 120 mM Tris-Cl, pH 6.8), resolved by SDS-PAGE and transferred with standard procedures.

Recombination assays. The DR-GFP reporter has been described previously26. The plasmid was integrated into the genome of MEFs via transfection with X-tremeGENE 9 (Roche), and colonies were selected in puromycin (2.5 μg/ml). Single-copy intact integration was confirmed by Southern blotting. I-SceI was transiently expressed via infection with I-SceI–encoding adenovirus (AdNGUS24i), and recombination frequency was determined as the percentage of GFP+ cells 3 d after infection. Background levels of GFP were determined by infection of cells with an empty adenovirus for comparison. Flow cytometry was carried out on an Accuri C6 Flow Cytometer (BD Biosciences) as previously described60. Data were analyzed with FlowJo.

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