Coordinated nuclease activities counteract Ku at single-ended DNA double-strand breaks

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Repair of single-ended DNA double-strand breaks (seDSBs) by homologous recombination (HR) requires the generation of a 3’ single-strand DNA overhang by exonuclease activities in a process called DNA resection. However, it is anticipated that the highly abundant DNA end-binding protein Ku sequesters seDSBs and shields them from exonuclease activities. Despite pioneering works in yeast, it is unclear how mammalian cells counteract Ku at seDSBs to allow HR to proceed. Here we show that in human cells, ATM-dependent phosphorylation of CtIP and the epistatic and coordinated actions of MRE11 and CtIP nuclease activities are required to limit the stable loading of Ku on seDSBs. We also provide evidence for a hitherto unsuspected additional mechanism that contributes to prevent Ku accumulation at seDSBs, acting downstream of MRE11 endonuclease activity and in parallel with MRE11 exonuclease activity. Finally, we show that Ku persistence at seDSBs compromises Rad51 focus assembly but not DNA resection.
ven a single DNA double-strand break (DSB) is a major threat to genome integrity, since it creates two new uncapped chromosomal DNA ends. In the absence of DNA repair, a DSB can lead to loss of a chromosome or chromosomal fragment and eventually cell death, while if inaccurately repaired, it can promote mutations or large DNA rearrangements such as translocations that can contribute to cell transformation. As part of genome maintenance mechanisms, two main pathways have evolved to repair DSBs in human cells: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ comprises direct ligation of the DSB ends and is initiated by the DNA termini being recognized by the Ku heterodimer, a highly abundant nuclear protein with strong affinity for a double-stranded DNA end that is threaded into its cavity. Ku bound at a DNA end then recruits the protein PAXX (refs 5,6) plus the catalytic subunit of the DNA-dependent protein kinase (DNA-PKs)7. The complex of Ku and DNA-PKcs at the DNA end forms the DNA-PK, a ser/threonine kinase able to phosphorylate various substrates and which regulates the action of CtIP and MRE11 nuclease activities. Although several factors such as nuclear architecture, chromatin and transcriptional context influence repair-pathway choice, NHEJ has the capacity to repair any DSB, providing that it comprises two DNA ends8–10. By contrast, end joining is not available as a mechanism of repair at single-ended DSBs (seDSBs), owing to the lack of another DNA end to be ligated to and therefore, seDSBs are preferentially repaired by HR. seDSBs can be generated during replication by the collision of progressing replication forks with various DNA lesions11. Repair by HR requires a homology template, most often the sister chromatid, thus helping to explain, why HR is restricted to S and G2 phases of the cell cycle. HR is initiated by DNA resection: the exonucleolytic processing of the 5′-end of the break to generate a free 3′-single-stranded overhang that is stabilized by it being coated with replication protein A, a heterotrimeric complex of RPA70, RPA32 and RPA14 (ref. 2). Notably, free double-stranded DNA ends in human cells are known to be rapidly bound by Ku, owing to its high abundance and affinity for DNA termini. In addition to promoting NHEJ, Ku also shields DNA ends from exonucleases12–15.

In the unicellular organisms Saccharomyces cerevisiae and Schizosaccharomyces pombe, the proteins Sae2 and SpCtp1 (homologues of the human CtIP protein) together with the MRE11–RAD50–Xrs2/NBS1 complex initiate DNA resection at DSB ends through MRE11 endonuclease activity creating an adjacent DNA nick that is then processed in the 5′–3′ and 3′–5′ directions by EXO1-DNA2 and MRE11 exonuclease activities, respectively16,17. The discovery of MRE11 endo- and exonuclease inhibitors has recently supported the existence of a similar mechanism in higher organisms18,19. This mode of ‘bi-directional resection’ thus helps explain how resection can be initiated in the presence of Ku, but still leaves the issue of how Ku is finally removed from DNA ends to allow HR to proceed. A role for MRE11 endonuclease activity in the process of Ku release has been demonstrated in yeast but remains to be tested in mammalian cells12,20–22. In addition, while parallels between this mechanism and Spo11 removal from meiotic DSBs have led to the proposal that eviction of Ku from the DNA end is performed by MRE11 exonuclease activity ‘pushing’ it away16, this idea remains to be experimentally tested both in yeast and in human cells.

Here, by using our recently published method to monitor Ku accumulation at DSBs23, we show that Ku indeed recognizes seDSBs in human cells. We also establish that ATM-dependent phosphorylation of CtIP plus MRE11 endonuclease activity counteract Ku accumulation at seDSBs. Furthermore, we show that downstream of MRE11 endonuclease activity, the epistatic action of MRE11 exonuclease activity and the recently discovered CtIP flap endonuclease activity24,25 are required to antagonize Ku, and also for efficient RAD51 loading at seDSBs. Our work also provides evidence for a hitherto unsuspected mechanism operating in parallel to MRE11 exonuclease and CtIP flap-endonuclease activities to counteract Ku persisting at seDSBs. We propose that MRE11 endonuclease and exonuclease activities process the DNA flanking Ku, and therefore prime Ku for release by CtIP 5′-flap endonuclease activity. We anticipate that ‘attacking DNA ends from the flanks’ through the coordinated action of CtIP and MRE11 nuclease activities is a general mechanism to repair complex DNA lesions congested with proteins or bulky DNA adducts.

**Results**

**Ku transiently binds to seDSBs.** To decipher the mechanisms regulating Ku binding to and persisting at seDSBs in human cells, we used the topoisomerase I (TopoI) inhibitor and anticancer agent camptothecin (CPT). By stabilizing covalent complexes of TopoI with DNA, CPT promotes the generation of seDSBs associated with replication forks (Supplementary Fig. 1).26,27 In agreement with previous findings28, we observed by immunoblotting that CPT treatment induced phosphorylation of RPA32 on Ser-4/Ser-8 (RPA32/S4/S8) in human U2OS cells in a manner that was abrogated when cells were co-treated with either the specific DNA-PK inhibitor NU7441 (DNA-PKsi)29 or with the DNA-replication inhibitor aphidicolin (Fig. 1a, note that phosphorylated KAP-1, an ATM target, is still generated in the presence of DNA-PK inhibitor, indicating that DNA-PK inhibition does not affect DSB induction per se). Because DNA-PK activity requires Ku binding to DSBs, these findings implied that an active DNA-PK complex, composed of the Ku70-Ku80 dimer and DNA-PKcs, assembles on and is activated by seDSBs generated by CPT. By using high-resolution imaging, we examined individual seDSBs marked by resection-dependent RPA70 foci and therefore directed towards HR-repair. This revealed that formation of RPA70 foci was accompanied by phosphorylation of S4/S8 RPA32 (RPA32/S4/S8) after CPT treatment (Fig. 1b), supporting the idea that seDSBs are initially recognized by Ku. In agreement with this, inclusion of DNA-PK inhibitor leads to a global decrease of P-RPAS4/S8 foci number and intensity (Fig. 1b–d). Importantly, DNA-PK inhibition had no significant effect on RPA32 foci formation in the same cells, indicating that single-strand DNA (ssDNA) production is essentially unaffected by DNA-PK inhibition (Fig. 1c).

To establish whether S4/S8 RPA32 phosphorylation is indeed a read-out of Ku binding to DNA ends, we replaced endogenous Ku70 by wild-type Ku70 or by the Mut6E mutant of Ku70, which has been shown to be unable to interact with DNA ends23 (Fig. 1e). Notably, compared with the wild-type control, replacing of Ku70 by the Mut6E mutant reduced the number and intensity of P-RPAS4/S8 foci number and intensity (Fig. 1e). Importantly, DNA-PK inhibition had no significant effect on RPA70 foci formation in the same cells, indicating that DNA resection is largely unaffected by the inability of Ku to bind DNA (Fig. 1f–h). Together, these data established that Ku binds to seDSBs generated by CPT treatment, promoting the assembly of a functional DNA-PK complex that mediates RPA32 phosphorylation on S4/S8 (Fig. 1i).

Next, we assessed whether Ku persists long enough on seDSBs to allow detection of Ku foci by high-resolution fluorescence microscopy. As published previously23, we observed accumulation of Ku (~250 Ku80 foci per nucleus; see ref. 23 for quantification) at sites of DNA damage caused by ionizing radiation but not at seDSBs induced by CPT (Fig. 1j). Collectively, these data suggested that Ku and DNA-
Figure 1 | RPA32 S4/S8 phosphorylation is a mark of transient Ku association with seDSBs. (a) Immunoblotting of extracts from U2OS cells pre-treated with the replication inhibitor aphidicolin (APH) or with DNA-PK inhibitor (DNA-PKi), respectively, before being CPT treated. (b) Representative micrographs of P-RPAS4/S8 and RPA70 foci detected by immunofluorescence in U2OS cells pre-treated with dimethylsulfoxide (DMSO) or DNA-PKi before being treated with CPT. After treatment, cells were pre-extracted with CSK + R (see Methods section) before fixation and immunodetection. (c) Quantification of RPA70 and P-RPAS4/S8 foci number per cell. Cells were treated and processed as in b, and foci quantified as described in Methods. (d) Graph representing the distribution of individual P-RPAS4/S8 focus intensity. Cells were treated and processed as in b. 6,684 and 8,462 foci were analysed for the CPT and CPT + DNA-PKi conditions, respectively. (e) Immunoblotting of extracts from U2OS T-REx cells stably transfected with control or siRNA-resistant wild-type (WT) or Mut6E GFP-FLAG-Ku70, and transfected with the indicated siRNA. (f) Representative micrographs of P-RPAS4/S8 and RPA70 foci detected by immunofluorescence in U2OS T-REx transfected as in e and treated with CPT. After treatment, cells were pre-extracted with CSK + R before fixation and immunodetection. (g) Quantification of RPA70 and P-RPAS4/S8 foci number per cell. Cells were treated and processed as in f and foci quantified as described in Methods. (h) Graph representing the distribution of individual P-RPAS4/S8 focus intensity in U2OS T-REx cells treated and processed as in f. 9,970 and 19,995 foci were analysed for the Ku70-WT and Ku70-Mut6E conditions, respectively. (i) Model depicting the proposed structure transiently forming at seDSB induced by CPT. (j) U2OS cells were treated with ionizing radiation (IR) or CPT before being pre-extracted with CSK + R and processed for immunodetection of Ku80 and the replication marker proliferating cell nuclear antigen (PCNA). White scale bars represent 10 μm; insets represent ×3 magnification. Error bars are s.d. Significant differences between specified pairs of conditions, as judged by t-test, are highlighted by stars (*P < 0.05). NS, non-significant difference.
PKcs load transiently on seDSBs as reported by DNA-PK-dependent phosphorylation of RPA32 but that activities prevent stable Ku binding to these DNA ends repaired by HR.

**CtIP prevents Ku accumulation at seDSBs.** Since a role for CtIP homologues scSae2 and spCtp1 in removing Ku from DSBs has been demonstrated in yeast12,20–22, we next used short-interfering RNA (siRNA) to deplete CtIP from human cells (Fig. 2a). As described previously29,30, CtIP depletion strongly impaired DNA resection as detected by a flow cytometry-based assay (Supplementary Fig. 2a,b)30 without affecting the amount of DSB induced by CPT as revealed by quantitative analysis of the DSB marker phosphorylated histone H2AX (γH2AX, Supplementary Fig. 2c). Strikingly, CtIP depletion also allowed accumulation of Ku foci in replicating cells treated with CPT (see Fig. 2b, right panel, and quantification, Fig. 2c). These findings thus supported there being a critical role for human CtIP in counteracting the accumulation of Ku on seDSBs, as has been reported in yeast for scSae2 and spCtp1 (refs 12,20–22). Importantly, we found that the sensitization to cell killing by CPT that is produced by CtIP depletion could be partly rescued by inhibition of DNA-PK (Fig. 2d), suggesting that toxic repair events mediated by NHEJ underlie this sensitization. Under these conditions, DNA-PK inhibition alone had no significant impact on survival of cells transfected with control siRNA, consistent with CtIP inducing DNA ends that are normally not repaired by NHEJ but by HR.

**ATM phosphorylates CtIP to prevent Ku persistence at seDSBs.** In agreement with our previous findings23 and through the use of the specific ATM inhibitor KU55933 (ref. 31), we found that ATM kinase activity also counteracts Ku at seDSBs, although to a lesser extent than CtIP depletion (Fig. 2c). Importantly, since...
The addition of ATM inhibitor did not further increase the number of Ku foci under conditions of CtIP depletion (Fig. 2c), our data strongly suggested that in this regard, ATM and CtIP function in the same pathway. These findings thus suggested that ATM prevents Ku accumulation on seDSBs by stimulating CtIP activity, likely through direct CtIP phosphorylation. To test this, we established a complementation system to express in CtIP-depleted cells, siRNA-resistant wild-type or mutated CtIP, the latter being Ser to Ala mutations in three of its main ATM-mediated phosphorylation sites (S664, S679 and S745 (refs 32,33); Fig. 2e,f). In accord with ATM-mediated CtIP phosphorylation being essential to antagonize Ku accumulation on seDSBs, unlike wild-type CtIP, the phosphorylation site (S->A) mutant CtIP was unable to counteract Ku focus formation in response to CPT (Fig. 2g). These data thereby suggested a model in which ATM-mediated CtIP phosphorylation serves to positively regulate the ability of CtIP to prevent Ku from remaining at seDSBs.

MRE11 nuclease mediates CtIP function in antagonizing Ku. CtIP has been proposed to function together with the MRE11–RAD50–NBS1 complex to promote MRE11 nuclease activity. We therefore tested the role of MRE11 nuclease activity in counteracting Ku accumulation on seDSBs via replacing endogenous MRE11 by the H129N mutant, which is inactivated for both endo- and exonuclease activities, or by the wild-type MRE11 protein as a control (Fig. 3a,b). In contrast to the wild-type protein, expression of nuclease-inactive MRE11 led to a defect of DNA resection, similar to that caused by CtIP depletion (Fig. 3c), and also to a strong accumulation of Ku foci after CPT treatment (Fig. 3d,e). Importantly, CPT-induced KAP-1 phosphorylation on S824, which is ATM-dependent (Supplementary Fig. 3a), was essentially the same in wild-type versus H129N MRE11 expressing cells (Supplementary Fig. 3b), indicating that ATM activation is not affected by the H129N mutation. These data thus demonstrated that MRE11 nuclease activity is critical to restrain Ku from accumulating at seDSBs generated by CPT and confirmed that it is not required for ATM activation. Furthermore, we found that MRE11 nuclease activity functions in the same pathway as CtIP, since depleting CtIP in cells expressing MRE11 H129N did not further increase the number of Ku foci detected on CPT treatment (Fig. 3f).

To address the respective contributions of MRE11 exonuclease and endonuclease activities in counteracting Ku accumulation at seDSBs, and since the scMRE11 H59S mutant is selectively deficient in exonuclease activity, we mutated His63, the corresponding residue in human MRE11 to Ser or Asn, and replaced endogenous MRE11 by the H129N mutant, which is inactivated for both endo- and exonuclease activities, or by the wild-type MRE11 protein as a control (Fig. 3a,b). In contrast to the wild-type protein, expression of nuclease-inactive MRE11 led to a defect of DNA resection, similar to that caused by CtIP depletion (Fig. 3c), and also to a strong accumulation of Ku foci after CPT treatment (Fig. 3d,e). Importantly, CPT-induced KAP-1 phosphorylation on S824, which is ATM-dependent (Supplementary Fig. 3a), was essentially the same in wild-type versus H129N MRE11 expressing cells (Supplementary Fig. 3b), indicating that ATM activation is not affected by the H129N mutation. These data thus demonstrated that MRE11 nuclease activity is critical to restrain Ku from accumulating at seDSBs generated by CPT and confirmed that it is not required for ATM activation. Furthermore, we found that MRE11 nuclease activity functions in the same pathway as CtIP, since depleting CtIP in cells expressing MRE11 H129N did not further increase the number of Ku foci detected on CPT treatment (Fig. 3f).

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Figure 3 | MRE11 nuclease activities control Ku accumulation at seDSB. (a) Schematic of MRE11 domains with the position of MRE11 H129 and H63 residues. (b) Immunoblotting of extracts from U2OS T-REx cells stably transfected with control or siRNA-resistant wild-type (WT) or H129N HA-MRE11-expressing plasmids and transfected with the indicated siRNA. (c) U2OS or U2OS T-REx cells complemented with MRE11 as in b were transfected with the indicated siRNA, treated with CPT and processed for analysis of DNA resection as monitored by measuring RPA32 association with chromatin using a flow cytometry assay. (d) Representative micrographs and quantification of Ku foci in replicating U2OS T-REx cells complemented with MRE11 as in b and treated or not with CPT before being processed for immunofluorescence. Replicating cells were identified using proliferating cell nuclear antigen (PCNA) staining. (e) Schematic of MRE11 domains with the position of MRE11 H129 and H63 residues. (f) Analysis by denaturing PAGE of the exonuclease activity of WT and mutants MRE11 on the probe depicted in i. (g) Alignment of scSae2 H59 with human MRE11 H63 revealing that amino acid H59 is conserved in humans and corresponds to H63. (h) Immunoblotting of extracts from U2OS T-REx cells stably transfected with control or siRNA-resistant WT, H63S or H63N HA-MRE11-expressing plasmids, and transfected with the indicated siRNA. (i) 5′ radio-labelled double-stranded DNA substrate used for in vitro nuclease assays. (j) Analysis by denaturing PAGE of the exonuclease activity of WT and mutants MRE11 on the probe depicted in i. (k) Quantification of nuclease activity in each condition relative to the MRE11 WT condition. (l) Immunoblotting of bead-associated complexes used for in vitro nuclease assays. (m) Quantification of Ku foci in replicating U2OS T-REx cells complemented by WT or mutant HA-MRE11 as in f and treated with CPT. Error bars are s.d. Significant differences between specified pairs of conditions, as judged by t-test, are highlighted by stars (**P<0.01; ***P<0.0005; ****P<0.0001). NS, non-significant difference.
While the high abundance of Ku and its strong affinity for DNA ends suggested that it could probably load on any DSB, including a seDSB, this idea is still debated. Here by showing that RPA32 phosphorylation on S4/S8 on CPT treatment depends on Ku indeed recognizes seDSBs induced by CPT. Second, since scSae2, the CtIP homologue in S. cerevisiae, has also been reported to display structure-specific endonuclease activity24,37. Such a mechanism would thereby promote efficient repair by HR through minimizing the size of the double-stranded DNA region produced by CtIP endonuclease activity, by generating a DNA structure that CtIP has been shown to cleave in vitro24,25 (Fig. 5e). Such a mechanism would thereby promote efficient repair by HR through minimizing the size of the double-stranded DNA region produced by CtIP endonuclease activity, by generating a DNA structure that CtIP has been shown to cleave in vitro24,25 (Fig. 5e). Such a mechanism would thereby promote efficient repair by HR through minimizing the size of the double-stranded DNA region produced by CtIP endonuclease activity, by generating a DNA structure that CtIP has been shown to cleave in vitro24,25 (Fig. 5e). Such a mechanism would thereby promote efficient repair by HR through minimizing the size of the double-stranded DNA region produced by CtIP endonuclease activity, by generating a DNA structure that CtIP has been shown to cleave in vitro24,25 (Fig. 5e).

**Figure 4 | CtIP 5′-flap endonuclease activity shows epistasis with MRE11 3′-5′ exonuclease activity in counteracting Ku binding to seDSBs.**

(a) Schematic of CtIP domains with the position of N289 and H290 residues. (b) Immunoblotting of extracts from U2OS T-REx cells stably transfected with control or siRNA-resistant wild-type (WT) or NAHA HA-CtIP-expressing plasmids, and transfected with the indicated siRNA. (c) Quantification of Ku focus number in replicating cells transfected as in b and treated with CPT. (d) Immunoblotting of extracts from U2OS T-REx cells stably transfected with control or siRNA-resistant WT HA-CtIP and HA-MRE11 or NAHA HA-CtIP and H63N HA-MRE11, and transfected with the indicated siRNAs. (e) Quantification of Ku foci in U2OS T-REx stably transfected with control or the specified siRNA-resistant constructs and transfected with the indicated siRNAs. Error bars are s.d. Significant differences between specified pairs of conditions, as judged by t-test, are highlighted by stars (*P<0.05; **P<0.01; ***P<0.0001; ****P<0.00001). NS, non-significant difference.

Our work therefore supports strong coordination between MRE11 endo- and exonuclease and CtIP endonuclease activities, a mechanism that is likely to be conserved in other organisms, since scSae2, the CtIP homologue in S. cerevisiae, has also been reported to display structure-specific endonuclease activity24,37. Contrasting with previous models wherein MRE11 exonuclease activity somehow pushes Ku away and off DNA ends44, our data support a model in which Ku affinity for DNA ends prevents its simple release through displacement from a subset of DNA ends and instead requires the elimination of a fragment of double-stranded DNA carrying Ku. The generation and fate of such DNA fragments clearly deserve further investigations.

Our work has also highlighted how ATM kinase activity is critical in limiting Ku residence at seDSBs through the phosphorylation of CtIP. How CtIP phosphorylation regulates CtIP activity is still unclear but our data are in agreement with recent work showing that CtIP endonuclease activity depends on ATM-dependent CtIP phosphorylation44. The function of ATM
in promoting HR-dependent DSB repair was already well documented\textsuperscript{45–48}, despite the fact that in mouse cells, the impact of ATM inhibition on HR repair is not equivalent to loss of ATM protein\textsuperscript{49,50}. Our work thereby further highlights how targeting ATM kinase could be used to increase the efficiency of CPT derivatives in anticancer treatments through blocking HR-dependent DNA repair processes.

While Ku binding to DNA DSBs represents the first step of NHEJ, our data indicate that it can also be regarded as the first step in HR, with Ku binding protecting DNA ends from unregulated processing and helping to create a situation in which signals are integrated into a 'decision' whether the break should be channelled into NHEJ or HR. Supporting this model, previous works in yeast have shown that Ku has a function at seDSBs, and...
more generally at DSBs, to regulate the timing of DNA-end processing through blocking access of certain factors to DNA ends\textsuperscript{13,31-34}. It seems likely that multiple signals promoting HR, such as cyclin-dependent kinase-mediated phosphorylations, the presence or absence of cohesin, chromatin state and nuclear architecture might depend on and/or cooperate with Ku to regulate the balance between NHEJ and HR\textsuperscript{10,53,56}. While DNA resection can be initiated and performed in the presence of Ku, our findings indicate that the replacement of RPA by RAD51 is impaire by the presence of Ku at the DNA end (Fig. 5c). This suggests that RPA-RAD51 exchange on ssDNA is coupled to the removal of Ku and/or the Ku-associated double-stranded DNA fragment from DNA ends. The mechanism for such coupling clearly deserves exploration through further studies.

Our work also opens new directions for research by providing evidence for an as-yet uncharacterized mechanism working downstream of MRE11 endonuclease activity—and in parallel to MRE11 exonuclease and CtIP flap-endonuclease activities—to mediate Ku release from more than half of ssDSBs (Fig. 4e). Several nuclease activities might substitute for MRE11 exonuclease and/or CtIP endonuclease functions. Alternatively, in some cases, Ku might be modified to loosen its affinity for DNA ends, perhaps as mimicked by separation-of-function mutants in yeast\textsuperscript{51} or degraded at ssDSB sites by mechanisms related to those shown to mediate Ku release from DNA after DSB repair is complete\textsuperscript{40,41}. In this regard, we note that the RNF138 E3 ubiquitin ligase has recently been shown to promote HR and to function in the same pathway as MRE11 to promote Ku release\textsuperscript{37,38}.

Finally, we suggest that coordination of MRE11 and CtIP nuclease activities is likely to operate at various DNA lesions, including ‘masked’ breaks generated by topoisomerase II inhibition, DNA breaks generated during meiosis by Spo11 nuclease and complex DSBs generated by heavy-particle irradiation. In all these cases, DNA end binding by the Ku protein is prevented by bulkiness or complexity at DNA ends, and NHEJ might be unable to proceed. Attacking the DNA ends ‘from the flank’ through MRE11 endonuclease activity may have evolved to deal with these types of DNA lesions, while the ability to cleave the second DNA strand when displacement of the end-blocking lesion is not possible might constitute a second system to free the end for productive repair. This second mechanism might be especially relevant in the repair of complex DNA DSBs that are resected independently of cell cycle phase\textsuperscript{59-61}.

**Methods**

**Cell culture.** U2OS and U2OS T-REX (from American Type Culture Collection and Thermo Fisher Scientific, respectively) were grown in a 5% CO\textsubscript{2} humidified incubator at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U ml\textsuperscript{-1} penicillin and 100 µg ml\textsuperscript{-1} streptomycin. Cells were routinely tested for mycoplasma contamination by DNA staining and microscopy, and mycoplasma-free cells were used in all experiments.

**siRNA transfection.** A list of siRNAs used in this work is provided in Supplementary Table 1. siRNA transfections were performed with Lipofectamine RNAiMAX (Thermo Fisher Scientific) using the manufacturer’s instructions. Final concentrations of siRNAs were 50 nM in each experiment. Where two siRNAs were used simultaneously, each was used at a concentration of 25 nM. For most experiments, two rounds of transfection were performed at 24 h intervals, and experiments were carried out 48 h after the second transfection. For experiments in GFP-FLAG-Ku70 expressing cells, previously published conditions were used\textsuperscript{31}.

**Plasmids.** All plasmids generated for complementation experiments are deposited on the Addgene plasmid repository together with fully annotated maps and sequences. Details about plasmid construction are provided in Supplementary Methods together with a table with the oligonucleotides used (Supplementary Table 2).

**Plasmid transfection and stable cell generation.** Plasmid transfections were carried out with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. For stable transfections, 5 µg of DNA and 10 µl of Lipofectamine 2000 were used to transfect 10\textsuperscript{5} U2OS T-REX cells seeded the day before in a 60 mm dish. The day after transfection, various cell dilutions were seeded into 140 mm dishes and puromycin was added at 0.25 µg ml\textsuperscript{-1} for selection. Two to three weeks afterwards, individual clones were isolated and screened.

**DNA damage and drug treatments.** DNA-PK inhibitor (NU7441) and ATM inhibitor (KU-55933), both from Tocris Bioscience, were used at 3 and 10 µM, respectively, with a pre-incubation period of 1 h. Aphidicolin (Sigma-Aldrich) was used at 10 µM with a pre-incubation period of 90 min. For inducing protein expression with pCE, doxycycline (Doxy, Clontech) was added at 2 µg ml\textsuperscript{-1} 24 h before treatment, and drug treatments were performed in presence of Doxy. CPT (Sigma-Aldrich) was used at 1 µM for 1 h. Ionizing radiation treatments correspond to a 10 Gy X-ray irradiation, using calibrated irradiators (RX-650; Faxitron) fitted with a 0.5 mm aluminum filter for soft X-rays, followed by a 5 min post-irradiation.

**Antibodies.** A list of all primary antibodies used in this work together with working conditions is provided in Supplementary Table 3.

**Immunoblotting.** For immunoblotting, whole-cell extracts were prepared by scraping cells in SDS-lysis buffer (SLB; 4% SDS, 20% glycerol and 120 mM Tris-HCl (pH 6.8), boiling 5 min at 95 °C and 10 strokes through a 25 G needle. Lysates were diluted to 5 µg ml\textsuperscript{-1} in SLB. For loading, an equal volume of a solution of 0.01% bromophenol blue and 200 mM dithiothreitol was added to the extracts which were boiled for 5 min at 95 °C. A unit of 40–50 µg of denatured proteins were loaded for each condition and separated on SDS pre-cast gradient 4–12% polyacrylamide TGE gels (BioRad) and transferred on to nitrocellulose membrane (Protran, Whatman). Peroxidase (Peroxidase, Epitope Biosciences) was used for detection (Odyssey, LI-COR Biosciences). Digital data were processed using Fiji\textsuperscript{62} and cropped using Photoshop CS6 (Adobe). Uncropped scans of the most important blots are provided in Supplementary Fig. 4.

**Immunofluorescence.** Cells were seeded on ~160 µm thick coverslips (VWR International) 24 h before experiments. After treatments, cells were pre-extracted by two incubation of 5 min at room temperature with CSK buffer (10 mM PIPES (pH 7.1), 100 mM NaCl, 300 mM sucrose, 3 mM Mg\textsubscript{2+} and 0.7% Triton X-100) containing 0.1% RNase A (CSK + R), except for RAD51 foci for which pre-extractions were performed once with CSK on ice for 5 min. After pre-extraction, cells were washed with PBS and fixed 15–20 min with 2% paraformaldehyde in PBS before being washed three times with PBS. Before staining, cells were permeabilized 5 min with PBS/0.2% Triton X-100, washed with PBS and blocked with PBS/0.1% Tween-20 (PBS-T) containing 5% bovine serum albumin (BSA). Coverslips were incubated 75 min with primary antibodies in PBS-T/5% BSA, then washed with PBS-T and incubated 45 min with appropriate goat secondary antibodies coupled to IRDye 800CW (LI-COR Biosciences). An infrared imager was used for detection (Odyssey, LI-COR Biosciences).

**High-resolution imaging using deconvolution.** High-resolution pictures were acquired by imaging z-stacks containing the whole cell with a 100× oil immersion objective of the Leica TCS SP8×8 confocal microscope (Leica Microsystems). Deconvolution and analysis were performed using SoftWoRx (Applied Precision) in conservative mode. On all pictures in the manuscript, the white scale bars correspond to 10 µm. For RPA70 and P-RRPAS/S8 staining, micrographs correspond to the projection of the maximum intensity of two adjacent slices, while for other staining they correspond to a single slice.

**Quantification of focus number per cell and focus intensity.** For Ku, P-RRPAS/S8, RPA70 and RAD51 foci quantification, cells were pre-extracted and processed for immunofluorescence. Deconvoluted pictures of > 10 cells were acquired for each condition and submitted to automated focus detection using the 3D iZi (Sigma-Aldrich) both with 10 and 300 pixels maximum size of foci, respectively, and with a threshold adjusted in each experiment using a positive control\textsuperscript{62,63}. To identify cells in S-phase, cells were cells were scored for overall phosphorylation.
co-stained for proliferating cell nuclear antigen, a protein that accumulates at chromatin in S-phase and persists after CSK co-stained for proliferating cell nuclear antigen, a protein that accumulates at...
50. Daniel, J. A. et al. Loss of ATM kinase activity leads to embryonic lethality in mice. J. Cell Biol. 198, 295–304 (2012).

51. Balestrini, A. et al. The Ku heterodimer and the metabolism of single-ended DNA double-strand breaks. Cell Rep. 3, 2033–2045 (2013).

52. Saad, H. et al. DNA dynamics during early double-strand break processing revealed by non-intrusive imaging of living cells. PLoS Genet. 10, e1004187 (2014).

53. Cheng, Q. et al. Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks. Nucleic Acids Res. 39, 9605–9619 (2011).

54. Krasner, D. S., Daley, J. M., Sung, P. & Niu, H. Interplay between Ku and replication protein A in the restriction of Exo1-mediated DNA break end resection. J. Biol. Chem. 290, 18860–188615 (2015).

55. Huertas, P. & Jackson, S. P. Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J. Biol. Chem. 284, 9558–9565 (2009).

56. Huertas, P., Cortes-Ledesma, F., Sartori, A. A., Aguilera, A. & Jackson, S. P. CDK targets Sac2 to control DNA-end resection and homologous recombination. Nature 455, 689–692 (2008).

57. Ismail, I. H. et al. The RNF138 E3 ligase displaces Ku to promote DNA end resection and regulate DNA repair pathway choice. Nat. Cell Biol. 17, 1466–1457 (2015).

58. Schmidt, C. K. et al. Systematic E2 screening reveals a UBE2D-RNF138-CtIP axis promoting DNA repair. Nat. Cell Biol. 17, 1458–1470 (2015).

59. Yajima, H. et al. The complexity of DNA double strand breaks is a critical factor enhancing end-resection. DNA Repair (Amst) 12, 936–946 (2013).

60. Barton, O. et al. Polo-like kinase 3 regulates CtIP during DNA double-strand break repair in G1. J. Cell Biol. 206, 877–894 (2014).

61. Quenet, V., Beucher, A., Barton, O., Takeda, S. & Lobrich, M. CtIP and MRN promote non-homologous end-joining of etoposide-induced DNA double-strand breaks in G1. Nucleic Acids Res. 39, 2144–2125 (2011).

62. Schindelin, J. et al. Fiji: an open-source platform for biomedical-image analysis. Nat. Methods 9, 676–682 (2012).

63. Bolte, S. & Cordelieres, F. P. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213–232 (2006).

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Author contributions

S.B. conceived the study and designed all experiments with input from S.P.J. and P. Calouz; S.B. and P. Chanut performed all experiments except when stated otherwise; J.C. helped with establishing stable cell lines and performed cell survival experiments; S.B., P. Calouz and S.P.J. wrote the manuscript.

Additional information

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Corrigendum: Coordinated nuclease activities counteract Ku at single-ended DNA double-strand breaks

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In this Article, the MRE11 exonuclease mutant H63D is consistently referred to incorrectly as H63N. These errors appear in the Results, Methods, Fig. 3, Fig. 4, Fig. 5, Supplementary Fig. 3, Supplementary Table 2 and Supplementary Methods. The authors sincerely apologize for this mistake.

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