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Dna2 initiates resection at clean DNA double-strand breaks

Sharad C. Paudyal[1], Shan Li[1], Hong Yan[2], Tony Hunter[3] and Zhongsheng You[1,*]

[1]Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA, [2]Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA and [3]Salk Institute, 10010 N Torrey Pines Road, La Jolla, CA 92037, USA

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ABSTRACT

Nucleolytic resection of DNA double-strand breaks (DSBs) is essential for both checkpoint activation and homology-mediated repair; however, the precise mechanism of resection, especially the initiation step, remains incompletely understood. Resection of blocked ends with protein or chemical adducts is believed to be initiated by the MRN complex in conjunction with CtIP through internal cleavage of the 5′ strand DNA. However, it is not clear whether resection of clean DSBs with free ends is also initiated by the same mechanism. Using the *Xenopus* nuclear extract system, here we show that the Dna2 nuclease directly initiates the resection of clean DSBs by cleaving the 5′ strand DNA ∼10–20 nucleotides away from the ends. In the absence of Dna2, MRN together with CtIP mediate an alternative resection initiation pathway where the nuclease activity of MRN apparently directly cleaves the 5′ strand DNA at more distal sites. MRN also facilitates resection initiation by promoting the recruitment of Dna2 and CtIP to the DNA substrate. The ssDNA-binding protein RPA promotes both Dna2- and CtIP–MRN-dependent resection initiation, but a RPA mutant can distinguish between these pathways. Our results strongly suggest that resection of blocked and clean DSBs is initiated via distinct mechanisms.

INTRODUCTION

DNA double-strand breaks (DSBs) are arguably the most hazardous forms of DNA damage in cells, which can be caused by ionizing radiation, reactive oxygen species, chemotherapeutic drugs and collapse of replication forks, or induced during genome engineering with CRISPR, ZFN and TALEN technologies (1–3). DSBs also occur as programmed recombination events during meiosis and V(D)J recombination in lymphocyte development (4,5). Regardless of their origin, DSBs pose a serious threat and can lead to genomic instability or cell death if not properly repaired. To cope with this problem, cells have evolved a highly sophisticated mechanism called DNA damage response (DDR) to detect, signal and repair these breaks (6–8). DSBs are repaired mainly by two largely competing pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) (9–11). While NHEJ can occur throughout the cell cycle, HR is mainly limited to S and G2 phases when a homologous copy of the damaged region is available (11–14). The choice between these repair pathways is dictated by end resection, a DNA processing mechanism that selectively degrades the 5′ strand DNA from the ends to generate long 3′ ssDNA overhangs required for HR in S and G2 phases of the cell cycle. By converting dsDNA ends into ssDNA structures, resection promotes HR and averts NHEJ (11,15–18). DSB resection also controls the checkpoint responses that coordinate DNA repair with other cellular processes such as cell cycle progression and gene expression (19–22). Checkpoint responses are controlled by ATM and ATR protein kinases, both of which are activated by DSBs (23–25). Whereas ATM activation occurs on double-strand DNA structure adjacent to the DNA break ends, ATR is activated on the ssDNA structure generated by resection (26–28). Consequently, DSB resection promotes the ATR checkpoint pathway and attenuates the ATM checkpoint pathway (29–31). Thus, resection governs both DNA repair and checkpoint signaling in the DSB damage response.

DSB resection involves multiple enzymatic activities including nucleases and helicases and is tightly regulated to ensure genomic stability (17,32). Studies in multiple organisms such as yeasts, *C. elegans*, *Xenopus laevis,* mice and humans have led to the proposal of a two-step, bi-directional model in which resection is initiated by cleavage of the 5′ strand DNA away from the DSB ends by MRN/MRX (Mre11, Rad50 and NBS1/XRS2) together with CtIP/Sae2 (functional ortholog of CtIP in budding yeast) (31,33–40). In this step, the endonuclease activity of the Mre11 subunit in MRN/MRX is believed to be responsible for the cleavage, although CtIP/Sae2 has also been suggested to con-

*To whom correspondence should be addressed. Tel: +1 314 362 9893; Fax: +1 314 362 7463; Email: zyou@wustl.edu

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tain endonuclease activity (33,34,41–45). In the subsequent stage of resection, the 3′ end at the cleavage site generated by initiation is then processed by MRN and Exd2 in the 3′-5′ direction (35,46,47), whereas the 5′ end is further resected by Exo1 (together with PCNA or the 9-1-1 complex) and Dna2 (together with BLM/WRN, RPA and Cdc24) in the 5′-3′ direction (33,34,48–64). The resulting long ssDNA overhangs then promote the activation of HR and the ATR checkpoint (10,11,28,29). The initial endocleavage step mediated by CtIP–MRN is essential for the resection of DSBs with protein or chemical adducts at the 5′ ends because the exonuclease or flap endonuclease activities in Exo1, Dna2, Exd2 and MRN cannot directly process these ends (65–72). Consistent with this notion, it has been shown that MRN/MRX and CtIP/Sae2 are absolutely required for the resection of Spo11-linked DSBs in meiosis and Topoisomerase II-linked DSBs in somatic cells and *Xenopus* extracts (43,66–74).

How resection of clean DSBs with free ends—which can be generated at collapsed replication forks or by endonucleases or cancer drugs—is initiated remains an outstanding question. Because Sae2–MRX in yeast can carry out limited resection of a clean DSB generated by HO endonuclease in the absence of the Exo1 and Dna2 pathways, it was suggested that resection of clean DSBs is also initiated by Sae2–MRX (CtIP–MRN) (33,34). However, this observation does not address which nuclease initiates resection of clean DSBs when all resection activities are present in cells. Unlike blocked DSBs, clean DSBs can be directly resected by Dna2 and Exo1 (together with other factors) *in vitro* (48,50–52,57,58,75), raising the possibility that these nucleases could initiate resection at these breaks. Consistent with this idea, it has been shown that Sae2 and MRX are not essential for resection and downstream HR at clean DSBs in yeast (76–79). In the *Xenopus* cytosolic extract the nuclease activity of MRN is dispensable for the overall resection of clean DSBs, which is in sharp contrast to 5′ blocked ends where the nuclease activity of MRN is absolutely essential (80). Likewise, the catalytic function of CtIP has also been shown to be dispensable for resection of clean DSBs in human cells (43). Furthermore, in reconstituted reactions with purified proteins, Sae2–MRX or CtIP–MRN, prefer blocked ends over free ends for 5′ strand cleavage (41,42). Together, these observations suggest the possibility that resection of clean DSBs is initiated by a different mechanism.

To dissect how resection is initiated at clean DSBs in the presence of all resection activities, we used the *Xenopus* nucleoplasmic extract (NPE) isolated from synthetic nuclei—a cell-free system that faithfully recapitulates the proper DNA damage response in S and G2 phases of the cell cycle (52,59,63,81–86). Our results indicate that like blocked DSBs, resection initiation of clean DSBs also occurs via endocleavage of the 5′ strand DNA; however, Dna2, but not CtIP–MRN, is the primary nuclease for this process. In the absence of Dna2 function, MRN together with CtIP initiate resection of clean DSBs via an alternative pathway where MRN cleaves the 5′ strand DNA at more distal sites. These two pathways of resection initiation can be distinguished by mutants of MRN and RPA.

MATERIALS AND METHODS

Xenopus nuclear extract, antibodies, immunodepletion, immunoblotting and co-immunoprecipitation

Xenopus nucleoplamic extract (NPE) was prepared from synthetic nuclei assembled in the crude egg extract as previously described (81). Dna2 antibody was raised in rabbits against a bacterially expressed His-tagged fusion protein containing the N-terminal 712 amino acids of *Xenopus* Dna2 protein. Antibodies against *Xenopus* Exo1, PCNA, RPA, CtIP, Mre11, WRN, NBS1 and Chk2 have been described before (26,31,52,63,87,88). For immunodepletion, 10 μl protein A agarose beads coupled with 50 μl of the protein antiserum or 50 μl each of two antisera for double-depletion were incubated with 50 μl NPE for 45 min at 4°C. Beads were then removed from the extract by low-speed centrifugation (5000 rpm) in a desktop microcentrifuge. The extract supernatant was then subjected to two additional rounds of depletion under the same conditions. To inhibit Mre11 nuclease activity, Mirin (Sigma-Aldrich) was added to the final concentration of 250 μM to untreated or depleted extract. Immunoblotting was performed using DyLight 800- and DyLight 680-conjugated secondary antibodies (Pierce) and an Odyssey Infrared Imaging System (LI-COR Biosciences), as described previously (52,85,86). To examine the interaction between RPA and Flag-Dna2 proteins shown in Figure 5E, Protein A agarose beads bound by RPA antibodies were incubated in NPE supplemented with recombinant Flag-Dna2(WT) or Flag-Dna2(27-1053) protein at 4°C for 1 h. The beads were then washed with 250 μl egg lysis buffer (ELB) containing 0.5% NP-40 for five times followed by elution of proteins with sample buffer and analyzed by western blotting.

Expression and purification of recombinant proteins

Baculoviruses expressing wild type, Flag-tagged *Xenopus* Dna2 (Flag-Dna2(WT)) were described previously (60). Flag-Dna2(D278A), Flag-Dna2(K655E) and Flag-Dna2(27-1053) expression constructs were generated by PCR and cloned into the pFastBac1 vector using a Gibson Assembly method. Flag-tagged *Xenopus* CtIP (Flag-CtIP) in the FastBac1 vector was kindly provided by Dr Jean Gautier (Columbia University) (69,89,90). All clones were verified by sequencing. Bacmids expressing CtIP and Dna2 proteins were generated in DH10Bac bacterial cells, and proteins were expressed in Sf9 cells using a Bac-to-Bac baculovirus expression system (Invitrogen), according to the manufacturer's protocol. The Dna2 and CtIP recombinant proteins were affinity-purified using anti-Flag M2 beads (Sigma) following the standard protocol (91). Purified proteins were aliquoted to 3 μl, frozen in liquid nitrogen and stored at –80°C. Expression and purification of wild-type (WT) MRN complex and a nuclease dead-mutant complex containing Mre11(H130N) were described previously (80,88). Expression and purification of WT RPA complex and the RPA (1NΔ) mutant complex were described previously (61). The RPA1 subunit in RPA (1NΔ) lacks 121 amino acids at the N-terminus (61).

DNA substrates and resection assays

A one-end biotinylated 2 kb DNA fragment with a clean DSB was generated by PCR with a 5′ biotinylated primer and pBluescript SK(–) as the template. The free 5′ end was ^{32}P-labeled with T4 polynucleotide kinase (PNK) in the presence of ^{32}P-γ-ATP. Another DNA substrate (2.1 kb) used in Supplementary Figure S1C and S1E was generated in the same way, but contains the DNA sequence that encodes the first 700 amino acids of human Exo1. To generate DNA substrates with a 5′ or 3′ overhang at both ends used in Supplementary Figure S2, a 2 kb PCR fragment derived from pBluescript SK(–) containing either KpnI or XhoI site on both ends was digested with KpnI or XhoI. The substrates were then treated with Alkaline Phosphatase (New England BioLabs) to dephosphorylate the ends followed by 5′ ^{32}P-labeling with PNK in the presence of ^{32}P-γ-ATP. Oligonucleotide marker was prepared by mixing 10–24, 30, 35, 40, 45 mer oligonucleotides derived from the sequence at the free 5′ end of the one-end biotinylated 2 kb DNA substrate used throughout the study. The marker mix was also 5′ ^{32}P-labeled using PNK. The 3′ ^{32}P-labeled, 6 kb dsDNA substrate used in the resection assay in Supplementary Figure S5B and D was prepared by digestion of the pRS315 plasmid with XhoI and labeled by end filling using exonuclease-deficient Klenow Fragment in the presence of ^{32}P-α-dCTP, dGTP, dTTP and ddATP, as described previously (52,85,86). The 5′ biotinylated, internally radiolabeled DNA substrate used in Supplementary Figure S3E and S3G was generated by PCR using 5′ biotinylated primers, and the template pBluescript SK(–) in the presence of ^{32}P-α-dCTP. The biotinylated substrate was then coupled to streptavidin magnetic beads (New England BioLabs) before being added to NPE for resection analysis.

A typical resection assay involved 10 μl NPE (treated or untreated) supplemented with an ATP regenerating system (2 mM ATP, 20 mM Phosphocreatine and 5 ng/μl Creatine Phosphokinase) and 1 μl (5 ng/μl) of a radiolabeled DNA substrate. After incubation at room temperature, 2 μl reactions were withdrawn and mixed with 10 μl stop buffer (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 0.2% bromophenol blue, 0.5% SDS, 80 mM Tris–HCl, pH 8.0) supplemented with 2 mg/ml Proteinase K. The samples were incubated at 37°C for 2 h, and then mixed with an equal volume of formamide, heated for 3 min at 95°C, chilled on ice and run on a pre-run 16% polyacrylamide–urea (8 M) gel. After running, the gel was incubated with a protein destaining buffer (40% methanol and 10% acetic acid) overnight with gentle shaking for fixation and removal of urea. The gel was then placed on top of DE-81 filter paper (to retain small DNA resection products) and Whatman filter paper and dried before autoradiography. The resection samples of 3′ ^{32}P-labeled, 6 kb dsDNA substrate were run on a 0.8% agarose gel followed by drying and autoradiography, as previously described (52,85,86). In order to determine whether resection of a clean DSB in the extract is initiated through clipping of the first nucleotide, a 5′ ^{32}P-labeled 2 kb DNA substrate was incubated with Lambda Exonuclease (New England BioLabs) to generate the radiolabeled mononucleotide, which was then used as a standard for comparison with the resection initiation products generated in the extract from the same substrate on a 20% polyacrylamide–urea gel.

To assay the flap endonuclease activity of recombinant Dna2 proteins, a flap DNA substrate was generated by annealing three ssDNA oligos (5′-CCA GTG AAT TCG AGC TCG GTA CCC GCT AGC GGG GAT CCT CTA-3′; 5′-^{32}P-ATT GGT TAT TTA CCG AGC TCG AAT TCA CTG G-3′; 5′-TAG AGG ATC CCC GCT AGC GGG-3′) as described previously (92). The 5′ end of the flap ssDNA was labeled with ^{32}P using PNK. 5′ ^{32}P-labeled oligos of 10–13 nucleotides in length derived from the same sequence of the ssDNA flap were used as markers. The flap substrate was incubated in reaction buffer (40 mM Tris, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, 2.5 mM DTT and 5% glycerol) along with recombinant Flag-Dna2 proteins at room temperature. The reaction samples were then treated the same way as resection reactions with 5′ ^{32}P-labeled dsDNA substrate except that a 20% polyacrylamide–urea gel was used.

DNA binding assay

For DNA binding assay in the extract, the one-end biotinylated 2 kb DNA fragment derived from pBluescript SK(–) was immobilized on streptavidin magnetic beads (New England BioLabs). 2.5 μl of beads coupled with 50 ng of biotinylated DNA fragment were incubated with 10 μl of NPE at room temperature for the indicated times. After incubation, beads were isolated by centrifugation and washed twice with 250 μl of egg lysis buffer. The beads were then treated with Lambda protein phosphatase (New England BioLabs) for 30 min at 30°C to dephosphorylate DNA-bound proteins (to avoid gel mobility changes that could affect accurate detection of the proteins on western blots). The DNA-associated proteins were then detected by western blotting.

RESULTS

Resection of clean DSBs is initiated through 5′ endocleavage, generating oligonucleotides

To determine how the resection is initiated at a clean DSB with a free 5′ end, we generated a one-end blocked 2 kb dsDNA fragment by PCR using a 5′ biotinylated primer. The other free 5′ end of the DNA fragment was labeled with ^{32}P using PNK followed by gel purification (Supplementary Figure S1A). Using this model substrate, we performed resection assays in *Xenopus* NPE, which has been used extensively for the study of the DNA end resection process (52,59,63,85,86). After incubating the DNA substrate in NPE, reactions were terminated at various time points, and resection initiation at the radiolabeled 5′ end was analyzed by resolving the resection products on 16% denaturing polyacrylamide gels. We found that initiation of resection at a clean DSB with blunt ends did not occur through clipping of the first nucleotide of the 5′ end (Supplementary Figure S1B). Rather, it took place by cleaving the 5′ strand DNA ∼10–20 nucleotides (nts) away from the end, generating oligonucleotides (Figure 1A) (due to its relatively large size, a portion of the original substrate was 'trapped' in the loading wells of the polyacrylamide gel). The kinetics

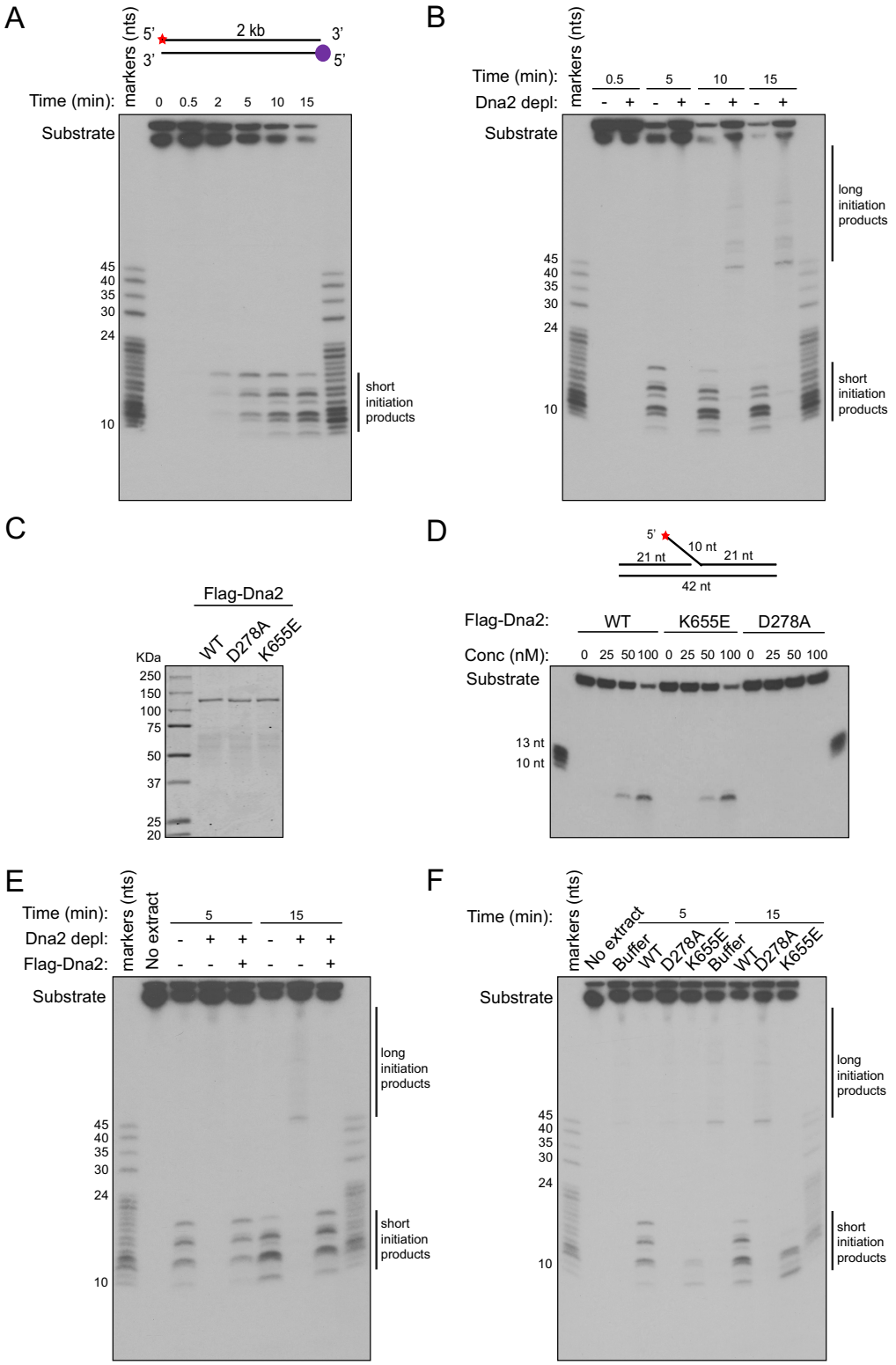


Figure 1. Dna2 initiates the resection of clean DSB ends via cleavage of 5′ strand DNA. (**A**) A one-end 5′ ^{32}P-labeled 2 kb DNA fragment (red star, ^{32}P; purple circle, biotin) was incubated in NPE at room temperature. Reactions were terminated at the indicated times and resection products were resolved on a 16% polyacrylamide-urea gel. The top band represents the original DNA substrate that was 'trapped' in the loading wells of the gel. (**B**) Effects of Dna2 depletion on the resection of the radiolabeled DNA substrate depicted in (A) in the extract. (**C**) Purified recombinant Flag-Dna2(WT), Flag-Dna2(D278A) and Flag-Dna2(K655E) expressed in insect cells. (**D**) Comparison of the flap endonuclease activity of Flag-Dna2(WT), Flag-Dna2(K655E) and Flag-Dna2(D278A) towards a dsDNA substrate with a 5′ ssDNA flap *in vitro*. (**E**) Rescue of short 5′ endocleavage products in the Dna2-depleted extract by recombinant Flag-Dna2(WT) protein. (**F**) Comparison of the ability of recombinant Flag-Dna2(WT), Flag-Dna2(K655E) and Flag-Dna2(D278A) in restoring resection initiation in the Dna2-depleted extract.

of the generation of these resection products suggests that multiple cleavage events occurred on the 5′ strand DNA at different locations. Incubation of another DNA substrate of similar length but with a different sequence also generated ssDNA oligos in a similar size range from the free 5′ end (Supplementary Figure S1C). Likewise, short oligonucleotides of ∼10–20 nts in length were also released from the 5′ ends of DNA substrates with 5′ or 3′ ssDNA overhangs in NPE (Supplementary Figure S2A), although the cleavage patterns differed significantly (Figure 1A, Supplementary Figures S1C and S2A). Together, these results suggest that resection of clean DSBs is mediated by internal cleavage of the 5′ strand DNA and that the cleavage sites are influenced by the end sequence and structure. To further dissect the mechanism of resection initiation at clean DSBs, we used primarily the one-end, 5′ ^{32}P-labeled 2 kb DNA fragment with blunt ends for subsequent studies.

Cleavage of free 5′ DNA ends at clean DSBs is mediated by Dna2

We next determined which nuclease(s) are responsible for the generation of the oligonucleotides from 5′ ends at clean DSBs. Although Sae2–MRX in yeast can initiate resection from clean DSBs in the absence of Dna2 and Exo1, it is not clear whether they also initiate resection at these breaks in the presence of the other resection activities in cells (33,34,41,42,76–78). It is possible that other resection nucleases such as Dna2 and Exo1 can directly initiate resection of these free DNA ends. To test this idea, we immunodepleted Dna2 or Exo1 from the NPE and examined the effects of depletion on the initiation of resection of the 2 kb DNA substrate with a blunt end described above. Depletion of Dna2 completely abrogated the generation of the 10–20 nts oligonucleotides from the radiolabeled 5′ end (Figure 1B, Supplementary Figure S6A). Depletion of Dna2 did not affect the proteins levels of MRN or CtIP (and vice versa) (Supplementary Figure S6E and H). To more rigorously demonstrate the role of Dna2 in resection initiation, we generated recombinant Flag-Dna2 in insect cells using the baculovirus expression system (Figure 1C). Addition of purified Flag-Dna2 to the Dna2-depleted extract rescued resection initiation (Figure 1E, Supplementary Figure S6B). Interestingly, we also observed longer oligonucleotides (≥45 nts) released from the 5′ end of the DNA substrate in the Dna2-depleted extract, which disappeared after addition of Flag-Dna2 (Figure 1B and E), suggesting that there exists an alternative mechanism for resection initiation in the absence of Dna2 (see below). Similar results were observed for other DNA substrates with blunt or recessed ends (Supplementary Figures S1D, S1E, S2B, S2C). In contrast to Dna2, depletion of Exo1 did not affect the production of the 5′ endocleavage products at a clean DSB, suggesting that Exo1 does not play a major role in resection initiation at clean DSBs (Supplementary Figure S3A and B).

Dna2 has both nuclease activity and helicase activity (93–100). To determine whether Dna2 directly initiates resection of clean DSBs, we tested the requirement of the nuclease activity of Dna2 for the endocleavage of 5′ ends. In contrast to WT Flag-Dna2, a nuclease-inactive mutant Flag-Dna2(D278A) failed to restore resection initiation in the Dna2-depleted extract (Figure 1C, F, Supplementary Figure S6C). This result strongly suggests that Dna2 directly initiates resection of clean DSBs through its 5′ flap endonuclease activity. In contrast to the nuclease-inactive mutant, the helicase-inactive mutant Flag-Dna2(K655E) was able to generate oligonucleotides from the free 5′ ends in the Dna2-depleted extract (Figure 1C, F, Supplementary Figure S6C). However, at an equal amount, Flag-Dna2(K655E) was much less efficient in initiating resection in the Dna2-depleted extract, compared to WT Flag-Dna2, although these proteins exhibited a similar level of 5′ flap endonuclease activity in reconstituted reactions (Figure 1D and F) (96,98,99). In addition, the 5′ oligonucleotides generated by Flag-Dna2(K655E) were significantly smaller in size, compared to that generated by WT Flag-Dna2 (Figure 1F). These results suggest that although the helicase activity of Dna2 is not essential for resection initiation at clean DSBs, it promotes Dna2 nuclease function and influences the cleavage sites on the 5′ strand DNA.

In the absence of Dna2, CtIP promotes resection initiation through endocleavage at more distal sites

In the Dna2-depleted extract we observed the release of resection products of 45 nts or longer from the radiolabeled 5′ end of the DNA substrate (Figure 1B, Supplementary Figures S1E and S2C). The appearance of these oligonucleotides was much delayed compared with the short oligonucleotides generated by Dna2 (Figure 1B, Supplementary Figure S1E). The long cleavage products were also observed in the extract containing Flag-Dna2(D278A) (Figure 1F). These observations suggest that there exists a backup mechanism to initiate the resection of clean DSBs ends in the absence of Dna2 or its nuclease activity. Depletion of Exo1 from the Dna2-depleted extract did not affect the production of these long 5′ oligonucleotides, indicating that Exo1 is not the nuclease that generates these products (Supplementary Figure S3C and D). Given the ability of MRX-Sae2 to initiate resection from HO cleavage-induced clean DSBs in yeast in the absence of Dna2 and Exo1 pathways (33,34), we hypothesized that CtIP–MRN is the alternative pathway that initiates resection of clean DNA ends in the Dna2-depleted extract. In support of this idea, we found that although depletion of CtIP alone had a very mild effect on the generation of the short oligonucleotide products by Dna2 (Figure 2A, Supplementary Figure S6D), co-depletion of CtIP from the Dna2-depleted extract completely abrogated the generation of the long oligonucleotides from the free 5′ end (Figure 2B, Supplementary Figure S6E). Addition of recombinant Flag-CtIP to the double-depleted extract restored the generation of long cleavage products (Figure 2C, D, Supplementary Figure S6F). We conclude that while Dna2 normally initiates resection from the free 5′ ends of DSBs, CtIP can mediate resection initiation at these ends when Dna2 is absent. This CtIP-mediated 'backup' mechanism also occurs through endocleavage, but at more distal sites from the DNA ends compared to the Dna2 cleavage sites.

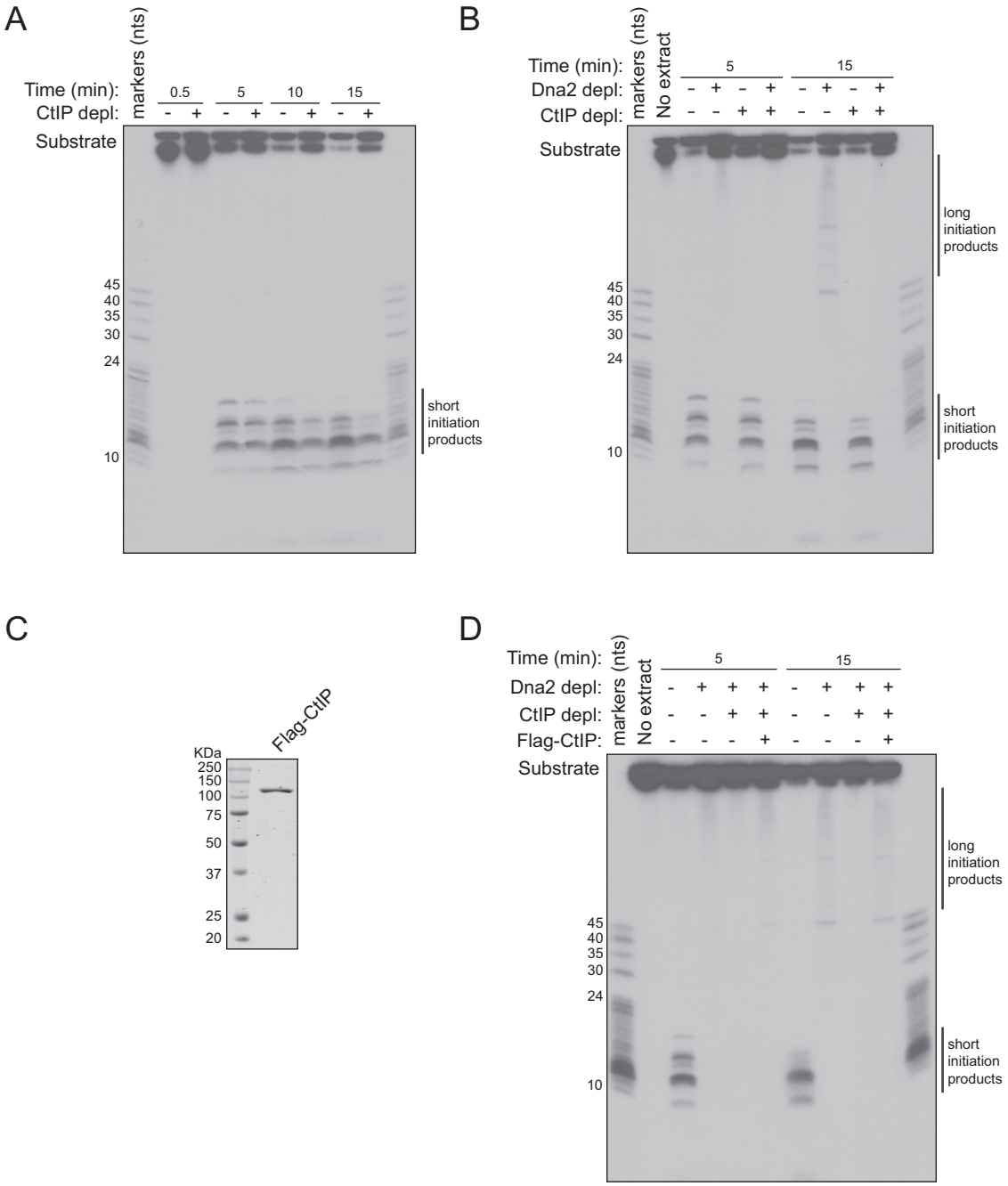


Figure 2. In the absence of Dna2, resection of clean DSBs is initiated through cleavage of 5′ strand DNA at more distal sites in a CtIP-dependent manner. (**A**) Effects of CtIP depletion on the initiation of resection of a 5′ radiolabeled clean DSB. (**B**) Effects of CtIP depletion on the generation of long endocleavage products in the Dna2-depleted extract. (**C**) Purified recombinant Flag-CtIP expressed in insect cells. (**D**) Rescue of long endocleavage products in the extract depleted of both Dna2 and CtIP by recombinant Flag-CtIP.

The nuclease activity of MRN is important for the CtIP-dependent pathway of resection initiation

We next asked whether MRN is the nuclease responsible for the CtIP-dependent pathway of resection initiation. Depletion of the Mre11 subunit of MRN from the extract did not affect Dna2 protein levels, but dramatically inhibited the generation of the short cleavage products, suggesting that MRN promotes Dna2-mediated resection initiation (Figure 3A, Supplementary Figure S6G). Mre11 depletion also abolished the long cleavage products in the presence or in the absence of Dna2, suggesting that MRN also promotes the CtIP-dependent initiation pathway (Figure 3A, B, Supplementary Figure S6G, S6H). To determine the potential role of the nuclease activity of MRN in the Dna2- and CtIP-dependent pathways, we first examined the effects of Mirin, an inhibitor of Mre11 nuclease activity, on resection initiation (46,101). Addition of Mirin to the extract did not affect the generation of short cleavage products (Figure 3C),

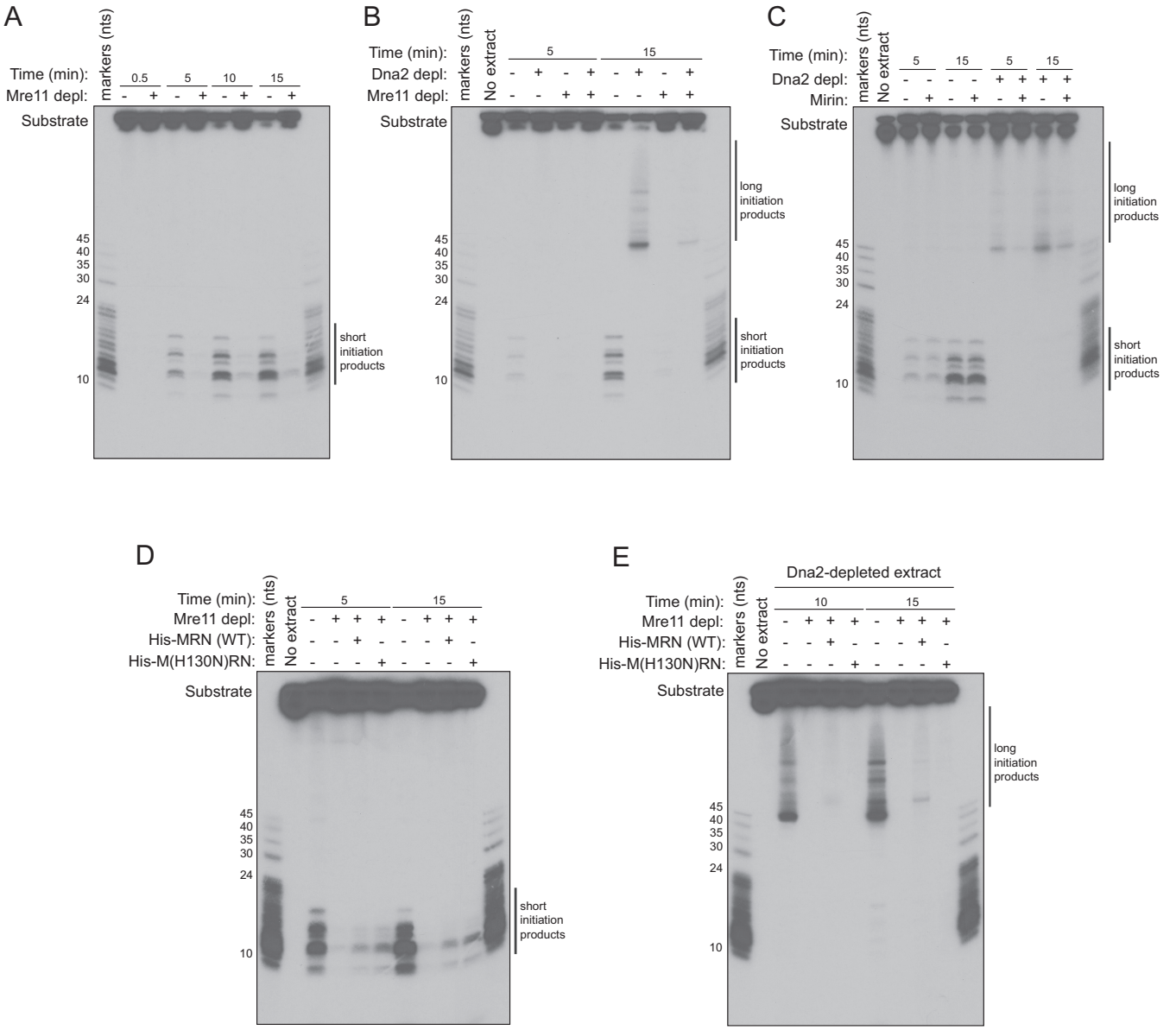


Figure 3. The nuclease activity of MRN is important for the CtIP-dependent pathway, but apparently not for the Dna2-dependent pathway. (**A**) Effects of Mre11 depletion on the generation of short resection initiation products in the extract. (**B**) Effects of Mre11 depletion on the generation of long resection initiation products in the Dna2-depleted extract. (**C**) Effects of Mirin on the generation of short and long resection initiation products in the extract. (**D**) Comparison of the ability of recombinant MRN(WT) and M(H130N)RN to rescue short resection initiation products in the Mre11-depleted extract. (**E**) Comparison of the ability of recombinant MRN(WT) and M(H130N)RN to rescue long resection initiation products in the extract depleted of both Dna2 and Mre11.

suggesting that the nuclease activity of MRN is not required for the Dna2 pathway of resection initiation. However, Mirin dramatically inhibited the generation of long cleavage products in the Dna2-depleted extract (Figure 3C, Supplementary Figure S6I), suggesting that the nuclease activity of MRN is responsible for the CtIP-dependent resection initiation pathway. To further test this idea, we compared the ability of WT or a nuclease-dead mutant of the MRN complex (M(H130N)RN) to support resection initiation. As shown in Figure 3D, WT MRN and M(H130N)RN both partially rescued short cleavage products generated by Dna2 to a similar extent in the Mre11-depleted extract suggesting that Mre11 nuclease activity is not required (due to the high concentration of MRN in NPE, we have not been able to generate recombinant MRN with a sufficiently high concentration/activity for full rescue, therefore at present we cannot rule out the possibility that another unknown factor that was co-depleted by the Mre11 antibodies also promotes Dna2-mediated cleavage.) (Figure 3D, Supplementary Figure S6J). In contrast, in the extract depleted of both Dna2 and Mre11, WT MRN, but not the nuclease-dead mutant, rescued long cleavage products, albeit partially (Figure 3E, Supplementary Figure S6K). This result is also consistent with the effects observed for Mirin described

above and further supports the idea that MRN is the nuclease that cleaves the 5′ strand DNA to initiate resection in the absence of Dna2. The nuclease activity of MRN is apparently dispensable for the Dna2 pathway, consistent with the idea that Dna2 itself is the responsible nuclease. Thus, MRN apparently plays distinct roles in the Dna2- and CtIP-dependent pathways of resection initiation.

MRN promotes the recruitment of Dna2 and CtIP to clean DSBs to initiate resection

To further characterize the role of MRN in the Dna2-dependent pathway of resection initiation at clean DSBs, we next determined whether MRN promotes the association of Dna2 with the DNA substrate. To this end, we disrupted MRN function in the extract by immunodepletion of NBS1 or by addition of previously characterized inhibitory antibodies of NBS1 (which inhibit the damage association of NBS1 in NPE as well as its phosphorylation by ATM in response to DSBs (Supplementary Figures S6L and S4A) (26,31,87). Consistent with the effects observed for Mre11 depletion, depletion or inhibition of NBS1 prevented the release of both short and long cleavage products from the 5′ end of the substrate in the presence or in the absence of Dna2 (Figure 4A–C, Supplementary Figure S6M). To assay the association of Dna2 with DNA substrate, we immobilized the 5′ biotinylated 2 kb DNA substrate on streptavidin beads and then incubated the DNA-bound beads with NPE. After incubation, the beads were pulled down and the proteins bound to the DNA substrate were detected by western blotting. As expected, Dna2 and other resection factors CtIP, NBS1 and Exo1 rapidly bound to the DNA substrate in NPE (Supplementary Figure S4B). Addition of inhibitory antibodies of NBS1 to NPE dramatically reduced the binding of Dna2 to the DNA substrate, suggesting that MRN promotes resection initiation by Dna2 by facilitating its damage recruitment (Figure 4D). Although we cannot completely rule out the possibility that the NBS1 antibodies affected the function of another factor in the extract that is important for Dna2 damage association, our result is consistent with the similar finding in yeast and in *in vitro* reconstituted reactions (51,57,102). Inhibition of NBS1 in the extract also abrogated the binding of CtIP in untreated extract or the Dna2-depleted extract (Figure 4D, E, Supplementary Figure S6N). NBS1 inhibition did not affect the initial binding of Exo1 or PCNA to the DNA substrate, although a mild decrease in damage association was observed at a later time point in the presence of the NBS1 antibodies (Figure 4D, E). Consistent with the multiple roles of MRN in DNA resection, disruption of NBS1 function inhibited the DNA-binding of RPA (Figure 4D and E). Depletion of Dna2 had no significant effects on the damage-association of NBS1 and CtIP (Supplementary Figure S4E), or the damage induced phosphorylation of NBS1 (Supplementary Figure S4C and D), further supporting that Dna2 functions downstream of MRN. Taken together, these results strongly suggest that in addition to its enzymatic role in the CtIP-dependent pathway, MRN facilitates the loading of both Dna2 and CtIP onto clean DSBs to promote resection initiation.

Interaction with RPA is important for Dna2-mediated resection initiation at clean DSBs

In addition to stabilizing the resulting 3′ ssDNA after resection, the ssDNA-binding protein RPA has also been shown to promote overall resection (51,57,58,60,61,75,103,104). However, its role in resection initiation is not understood. In reconstituted reactions with purified proteins, RPA promotes Dna2-mediated resection specifically on the 5′ strand DNA at DSBs (51,57,58,105). RPA directly associates with Dna2, which is mediated by the interaction between the N-terminus of Dna2 and the N-terminus of RPA1, the largest subunit of RPA (60,61,105). To determine whether RPA is required for Dna2-mediated initiation of resection at a clean DSB, we depleted RPA from NPE and then examined the effects on resection initiation at the clean DSB of the 2 kb DNA substrate used before. Depletion of RPA completely abrogated short cleavage products generated by Dna2 (Figure 5A, Supplementary Figure S6O). Importantly, this effect could be rescued by the addition of purified WT RPA complex (Figure 5I, Supplementary Figure S6S), indicating that RPA is required for Dna2-mediated pathway of resection initiation at clean DSBs.

To further investigate the role of RPA in resection initiation, we generated a Flag-Dna2(27–1053) mutant lacking the N-terminal 26 amino acids (Figure 5C). Consistent with the observation that the corresponding N-terminal region in mouse Dna2 interacts with RPA *in vitro* (105), Flag-Dna2(27–1053) exhibited reduced association with RPA in the extract compared to WT Flag-Dna2 (Figure 5E). Compared to WT Flag-Dna2, Flag-Dna2(27–1053)—which exhibited the same level of flap endonuclease activity *in vitro*—was significantly less efficient in cleaving free 5′ ends in the extract, suggesting that the interaction with RPA promotes Dna2-mediated resection initiation (Figure 5D, F, Supplementary Figure S6Q). In further support of this idea, a Dna2 binding-deficient RPA(1NΔ) mutant complex containing a N-terminally truncated RPA1 subunit could not rescue Dna2-mediated resection initiation in the RPA-depleted extract (Figure 5I, Supplementary Figure S6S), although this mutant retains DNA–binding activity (61). Depletion of RPA had only a very modest effect on the association of Dna2 with the DNA substrate (Figure 5G), consistent with the notion that RPA stimulates the nuclease activity of Dna2 towards 5′ ssDNA flaps after DNA unwinding (51,57,58,105).

RPA also promotes the CtIP–MRN-mediated resection initiation in the absence of Dna2

Depletion of RPA from the extract also abolished long endocleavage products (Figure 5A), raising the possibility that RPA is required for the CtIP–MRN-mediated 'backup' pathway of resection initiation. As the CtIP–MRN pathway initiates resection of clean DSBs in the absence of Dna2 function, we further examined the role of RPA in resection initiation in the Dna2-depleted extract. In contrast to the Dna2-depleted extract, no long cleavage products were generated in the extract depleted of both Dna2 and RPA (Figure 5B, Supplementary Figure S6P). This effect could be rescued by the addition of purified WT RPA complex (Figure 5J, Supplementary Figure S6T). The absence of long

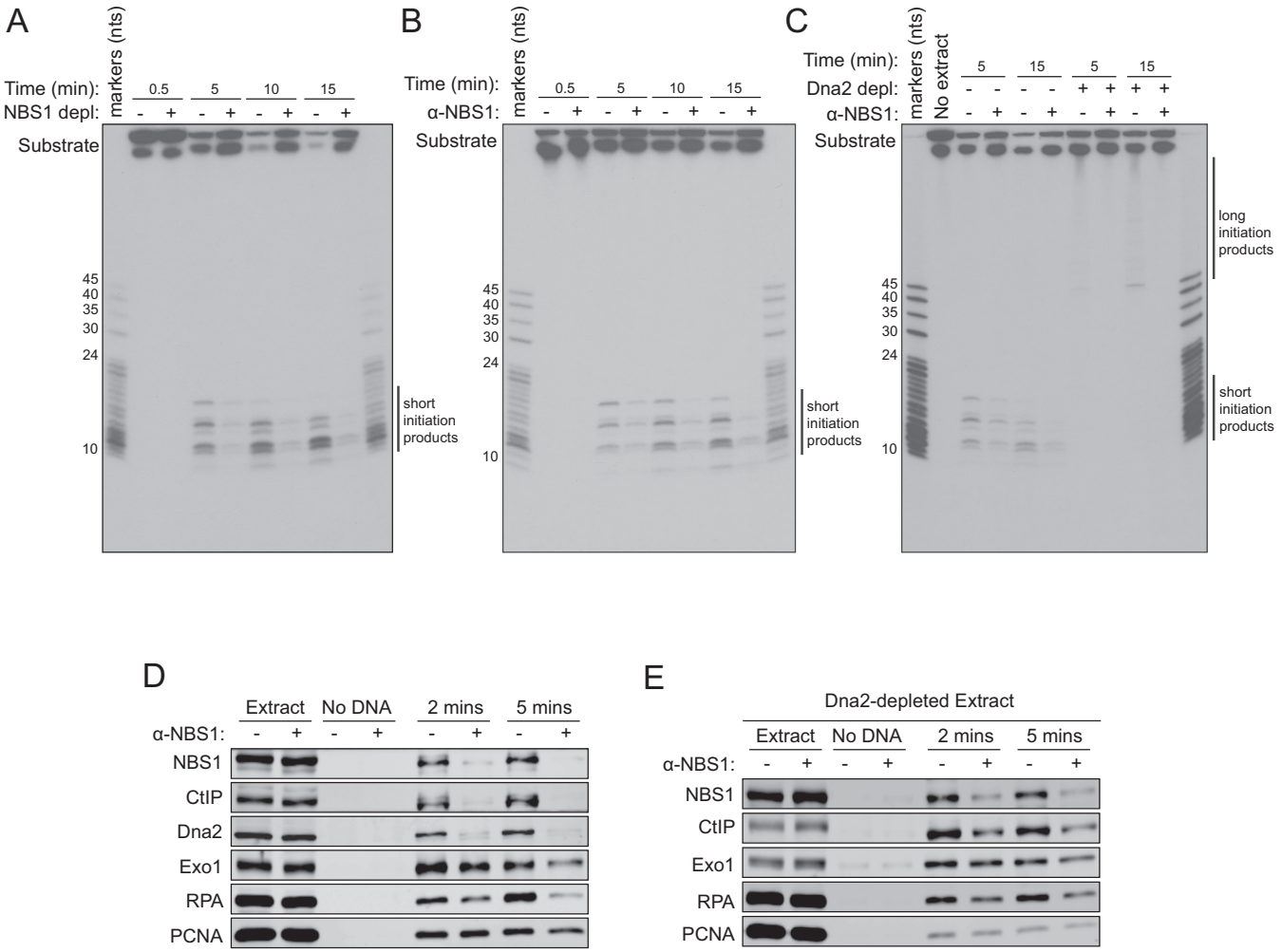
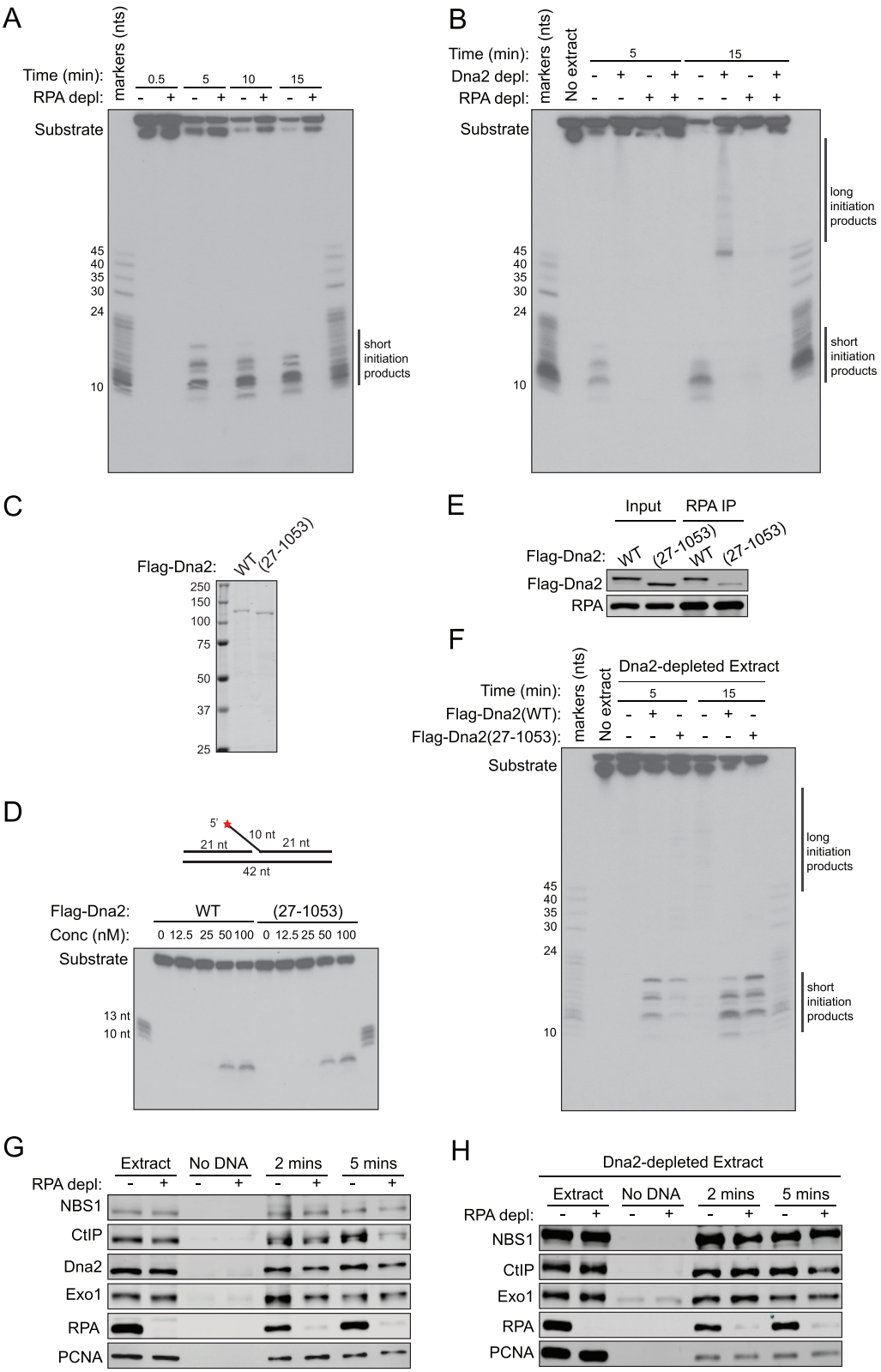


Figure 4. MRN promotes the recruitment of Dna2 and CtIP to DNA substrate to initiate resection. (**A**) Effects of NBS1 depletion on the generation of short resection initiation products in the extract. (**B**) Effects of NBS1 inhibitory antibodies on the generation of short resection initiation products in the extract. (**C**) Effects of NBS1 inhibitory antibodies on the generation of long resection initiation products in the Dna2-depleted extract. (**D**) Effects of NBS1 inhibitory antibodies on the binding of NBS1, CtIP, Dna2, Exo1, RPA and PCNA to the DNA substrate in the extract. (**E**) Effects of NBS1 inhibitory antibodies on the binding of NBS1, CtIP, Exo1, RPA and PCNA to the DNA substrate in the Dna2-depleted extract.

resection initiation products in the double-depleted extract was not simply caused by their degradation after endocleavage, because resection initiation was inhibited in the depleted extract and because no small degradation products were observed in the sample (Figure 5J). Together, these data strongly suggest that RPA also plays a critical role in the CtIP–MRN pathway of resection initiation. Interestingly, addition of the RPA(1NΔ) mutant complex to the extract depleted of both Dna2 and RPA could still rescue CtIP-dependent resection initiation (Figure 5J). This is in sharp contrast to its inability to support the Dna2 pathway (Figure 5I). These results suggest that RPA(1NΔ), similar to M(H130N)RN, is a separation-of-function mutant that can distinguish between these two resection initiation pathways. Depletion of RPA only modestly inhibited the binding of CtIP and NBS1 to the DNA substrate in the presence or in the absence of Dna2 (Figure 5G, H, Supplementary Figure S6R), indicating that RPA plays a minor role in the damage recruitment of these proteins.

DISCUSSION

Using a *Xenopus* nuclear extract system, we have investigated specifically the mechanism of resection initiation at clean DSBs and identified Dna2 as the primary nuclease for this step (Figure 1, Supplementary Figures S1 and S2). The MRN complex is critical for the Dna2-mediated resection initiation, but it apparently plays a nonenzymatic role by promoting the association of Dna2 with DNA substrate (Figures 3 and 4). In the absence of Dna2, the CtIP–MRN pathway operates as a backup pathway to initiate resection at clean DSBs, with MRN providing the nuclease activity (Figures 2 and 3). MRN also promotes CtIP recruitment to DNA substrate (Figure 4). Both the Dna2- and CtIP–MRN-dependent pathways initiate resection through endonucleolytic cleavage of 5′ strand DNA; however, the cleavage sites differ, with the CtIP–MRN pathway cleaving at more distal positions from the end compared to the Dna2 pathway (Figures 1 and 2). The ssDNA-binding protein RPA also promotes both resection initiation pathways,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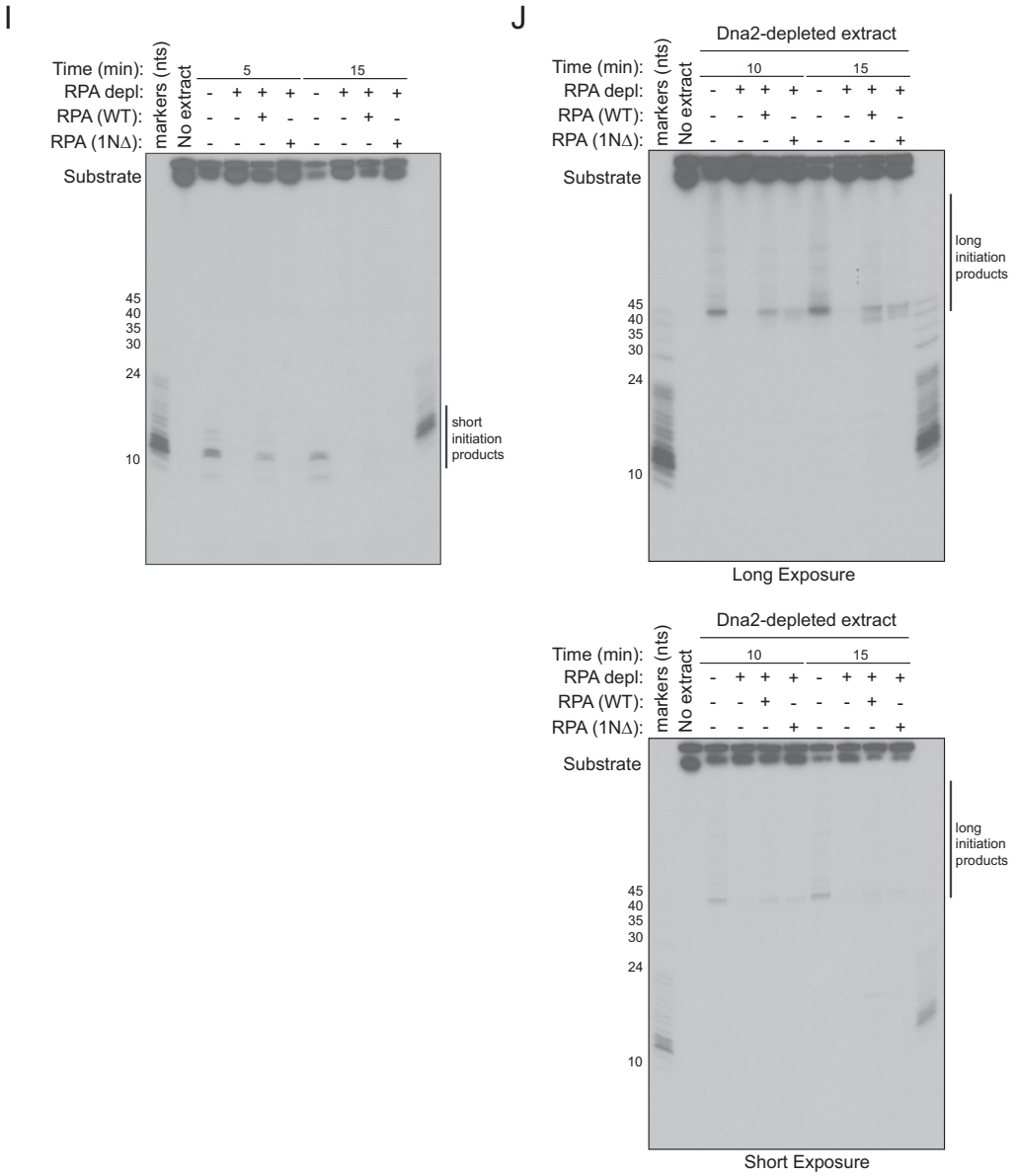

Figure 5. RPA plays a key role in resection initiation by both the Dna2- and CtIP-dependent pathways. (**A**) Effects of RPA depletion on the generation of short resection initiation products in the extract. (**B**) Effects of RPA depletion on the generation of long resection initiation products in the Dna2-depleted extract. (**C**) Purified recombinant Flag-Dna2(WT) and Flag-Dna2(27–1053) expressed in insect cells. (**D**) Comparison of the flap endonuclease activity of Flag-Dna2(WT) and Flag-Dna2(27–1053) *in vitro*. (**E**) Co-immunoprecipitation of RPA with Flag-Dna2(WT) and Flag-Dna2(27–1053) added to the extract. (**F**) Comparison of the ability of Flag-Dna2(WT) and Flag-Dna2(27–1053) to rescue short resection initiation products in the Dna2-depleted extract. (**G**) Effects of RPA depletion on the binding of NBS1, CtIP, Dna2, Exo1 and PCNA to the DNA substrate in the extract. (**H**) Effects of RPA depletion on the binding of NBS1, CtIP, Exo1 and PCNA to the DNA substrate in the Dna2-depleted extract. (**I**) Comparison of the ability of RPA(WT) and RPA(1NΔ) to rescue short resection initiation products in the RPA-depleted extract. (**J**) Comparison of the ability of RPA(WT) and RPA(1NΔ) to rescue long resection initiation products in the extract depleted of both Dna2 and RPA.

but it plays a minor role in facilitating the damage association of Dna2, CtIP and MRN (Figure 5).

The new model described above for resection initiation at clean DSBs is distinct from that for blocked DSBs with protein or chemical adducts (Figure 6). In budding yeast, Sae2–MRX can initiate resection of a HO-induced clean DSB in the genetic background deficient of the Dna2 and Exo1 resection pathways (33,34), indicating that Sae2–MRX (CtIP–MRN) has the ability to initiate resection from clean DSBs under this condition. However, this observation does not address whether or not Sae2–MRX (CtIP–MRN) normally initiates resection of clean DSBs when the Dna2 and Exo1 resection activities are present. Our results strongly suggest that the nuclease activity of Dna2, but not that of MRN, directly initiates resection of clean DSBs. Dna2 does so by cleaving 5′ strand DNA internally via its flap endonuclease activity (which requires free 5′ ends), generating short oligonucleotides (Figure 1). This is in sharp contrast to blocked DSBs whereby Sae2–MRX (CtIP–MRN) is believed to be the primary mechanism for

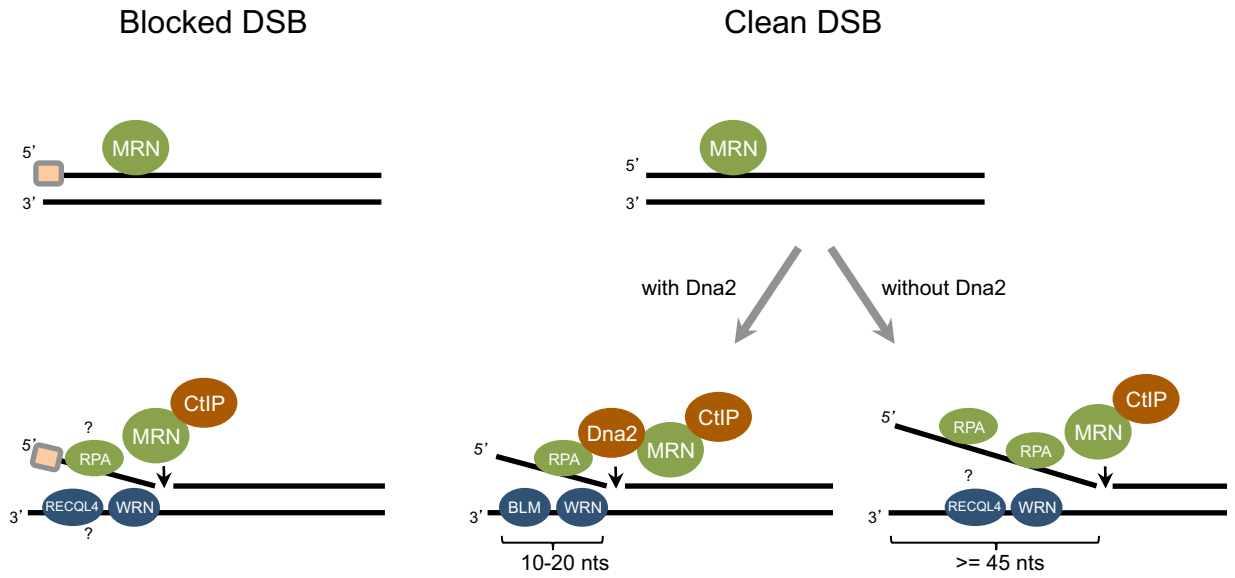

Figure 6. A model for resection initiation at clean or blocked DSBs. DNA end resection at a blocked DSB with protein or chemical adducts (denoted by pink rectangle) is initiated through endocleavage by the MRN nuclease in conjunction with CtIP, with the nuclease activity of MRN being responsible for the cleavage. By contrast, resection initiation at a clean DSB with free DNA ends is initiated by Dna2, which cleaves the 5′ strand DNA ∼10–20 nts away from the end. The MRN complex promotes this step by facilitating the recruitment of Dna2 to the DNA end. In the absence of Dna2, CtIP–MRN mediate an alternative pathway of resection initiation, with the endonuclease activity of MRN cleaving the 5′ strand DNA ≥45 nts away from the end. Both Dna2 and CtIP–MRN pathways of resection initiation apparently require DNA helicases such as WRN, BLM, RECQL4 and likely Dna2, which unwind DNA ends to generate ssDNA for cleavages. The ssDNA-binding protein RPA binds the resulting ssDNA and promotes resection initiation by both pathways. Dna2-mediated end cleavage requires the direct interaction between Dna2 and the N-terminus of RPA1 in the RPA complex, but this domain of RPA1 is dispensable for the CtIP–MRN pathway of resection initiation.

resection initiation (35,65–72). Consistent with this notion, we observed that resection of DSB ends blocked with biotin-streptavidin was initiated by the CtIP-dependent pathway, generating long cleavage products first (Supplementary Figure S3E-S3G). This observation is also in agreement with the finding that purified Sae2–MRX or CtIP–MRN prefers blocked DNA ends over free ends to initiate the resection in reconstituted reactions (41,42). The existence of the CtIP–MRN 'backup' pathway for resection initiation in the Dna2-depleted *Xenopus* extract also explains the observed limited resection by Sae2–MRX in the absence of the Dna2 and Exo1 pathways in yeast (33,34). It is important to point out that although not normally involved in resection initiation at clean DSBs, CtIP, like Dna2, contributes to overall resection of these breaks, presumably due to its role in resection extension (Supplementary Figure S5A–D) (31,33,34,48,88,90).

The generation of oligonucleotides from 5′ ends by both Dna2 and CtIP–MRN and the requirement of the ssDNA-binding protein RPA for both of these pathways suggest that helicase activity is required for resection initiation at clean DSBs. Indeed, we found that depletion of WRN helicase abrogated the short oligonucleotides generated by Dna2 (Supplementary Figure S5E, S5F), consistent with the previous finding that Dna2 acts in conjunction with WRN (or BLM) in resection (55,63,98,106). Interestingly, depletion of WRN from the extract also dramatically inhibited the generation of long cleavage products in the presence or in the absence of Dna2 (Supplementary Figure S5E and F), suggesting that WRN is also involved in the CtIP–MRN pathway of resection initiation. A recent study has shown that the helicase activity of RECQL4 is required for CtIP–MRN-dependent resection in human cells (107). Future studies are needed to further define the potential role of WRN and RECQL4 in CtIP–MRN-mediated resection initiation at clean DSBs. Dna2 itself also exhibits helicase activity, although its function is less understood compared to its nuclease activity (55,95,96,98,99,108,109). Interestingly, we found that a mutation that inactivates the helicase activity of Dna2 partially reduced the efficiency of resection initiation and affected the pattern of 5′ endocleavage (Figure 1F). While this manuscript was in preparation, two studies by the Sung group and Cejka group reported similar findings for yeast and human Dna2 whereby the helicase-deficient mutants also produce smaller resection products with a lower efficiency (110,111). These observations support the idea that although the helicase function of Dna2 is not essential for DSB resection, it promotes proper resection.

Our study has provided critical new insights into the function of the MRN complex in the resection of clean DSBs. While the overall resection of clean DSBs is less efficient in the absence of MRX in yeast, this complex is not absolutely essential for resection of these ends or downstream HR repair (76–78). This is in sharp contrast with 5′ blocked DSBs with chemical or protein adducts where resection cannot occur without MRX/MRN, as the nuclease activity directly initiates resection from these ends (35,65–72). Our results indicate that although MRN promotes resection initiation at clean DSBs, its role is to facilitate the damage recruitment of Dna2 that provides the nuclease activity for resection initiation, although we cannot rule out the possibility that

MRN also stimulates the nuclease activity of Dna2 (Figures 3A and 4A, B, D). Consistent with its nonenzymatic role in the Dna2 pathway, a low level of resection initiation was still observed in the Mre11- or NBS1-depleted extracts (Figures 3 and 4). However, the nuclease activity of MRN is apparently responsible for the CtIP-dependent backup pathway of resection initiation, which normally does not operate in the presence of Dna2 (Figure 3). Although CtIP has also been shown to contain endonuclease activity (43–45), recent studies by the Cejka group suggest that Mre11, but not CtIP, confers the nuclease activity in the CtIP–MRN functional unit for 5′ end cleavage (41,42). The presence of Dna2 prevents CtIP-mediated end cleavage; however, Dna2 apparently does not prevent the loading of CtIP onto the DNA substrate (Supplementary Figure S4E). Because the CtIP–MRN pathway is operational in the presence of a nuclease-inactive Dna2 mutant (Figure 1F) and because the CtIP–MRN-mediated resection initiation has a slower kinetics compared to the Dna2-pathway (Figure 1B), we suggest that the fast engagement of Dna2 with the DNA substrate followed by immediate resection initiation precludes the action of the CtIP–MRN pathway. The physiological significance for the existence of this CtIP–MRN back-up pathway for initiating resection at clean DSBs remains to be determined.

This study has also shed new light on the role of the ssDNA-binding protein RPA in DSB resection. RPA is known to promote both Dna2 and Exo1 resection pathways (51,57,58,60,61,75,103–105); however, its function in resection initiation was unclear. Our data indicate that RPA is important for both the Dna2 and CtIP–MRN pathways of resection initiation at clean DSBs (Figure 5). The requirement of RPA in CtIP–MRN-mediated resection initiation is surprising, as Sae2–MRX or CtIP–MRN can directly clip 5′ strand DNA at blocked DSBs in the absence of RPA in reconstituted reactions (41,42). However, a role of RPA in the CtIP–MRN-mediated resection initiation is consistent with the requirement of DNA helicases such as WRN (and potentially RECQL4) for the pathway (Supplementary Figure S5F) (107). RPA may stimulate the activity of CtIP–MRN or help to present ssDNA substrate after end unwinding for CtIP–MRN-mediated cleavage. Using a heat-inducible degron system to deplete RPA from budding yeast, Symington and colleagues previously observed limited resection by Sae2–MRX at a clean DSB generated by the HO endonuclease in the td-RFA1 strain (103). This Sae2–MRX-mediated residual resection could result from incomplete depletion of RPA before break generation by HO, as pointed out by the authors (103). Alternatively, this discrepancy in RPA requirement could reflect the mechanistic differences in resection initiation between yeast and metazoans. Although RPA is required for resection initiation at clean DSBs, a RPA(1NΔ) mutant complex could support resection initiation by CtIP–MRN, but not by Dna2, suggesting that RPA(1NΔ), like M(H130N)RN, can distinguish between these pathways. The identification of these separation-of-function mutants provides a unique opportunity for further elucidation of the two resection initiation mechanisms at clean DSBs in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Mehta,A. and Haber,J.E. (2014) Sources of DNA double-strand breaks and models of recombinational DNA repair. *Cold Spring Harb. Perspet. Biol.*, **6**, a016428.
2. Zeman,M.K. and Cimprich,K.A. (2014) Causes and consequences of replication stress. *Nat. Cell Biol.*, **16**, 2–9.
3. Kim,H. and Kim,J.S. (2014) A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.*, **15**, 321–334.
4. Lam,I. and Keeney,S. (2015) Mechanism and regulation of meiotic recombination initiation. *Cold Spring Harb. Perspet. Biol.*, **7**, a016634.
5. Schatz,D.G. and Swanson,P.C. (2011) V(D)J Recombination: mechanisms of Initiation. *Annu. Rev. Genet.*, **45**, 167–202.
6. Ciccia,A. and Elledge,S.J. (2010) The DNA damage response: making it safe to play with knives. *Mol. Cell*, **40**, 179–204.
7. Jackson,S. and Bartek,J. (2009) The DNA-damage response in human biology and disease. *Nature*, **461**, 1071–1078.
8. Polo,S. and Jackson,S. (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev.*, **25**, 409–433.
9. Kass,E.M. and Jasin,M. (2010) Collaboration and competition between DNA double-strand break repair pathways. *FEBS Lett.*, **584**, 3703–3708.
10. Sung,P. and Klein,H. (2006) Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nat. Rev. Mol. Cell Biol.*, **7**, 739–750.
11. Symington,L.S. and Gautier,J. (2011) Double-strand break end resection and repair pathway choice. *Annu. Rev. Genet.*, **45**, 247–271.
12. Branzei,D. and Foiani,M. (2008) Regulation of DNA repair throughout the cell cycle. *Nat. Rev. Mol. Cell Biol.*, **9**, 297–308.
13. Orthwein,A., Noordermeer,S.M., Wilson,M.D., Landry,S., Enchev,R.I., Sherker,A., Munro,M., Pinder,J., Salsman,J., Dellaire,G. *et al.* (2015) A mechanism for the suppression of homologous recombination in G1 cells. *Nature*, **528**, 422–426.
14. Rothkamm,K., Krüger,I., Thompson,L.H. and Lobrich,M. (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol. Cell. Biol.*, **23**, 5706–5715.
15. Paudyal,S.C. and You,Z. (2016) Sharpening the ends for repair: mechanisms and regulation of DNA resection. *Acta Biochim. Biophys. Sin. (Shanghai).*, **48**, 647–657.
16. Daley,J.M., Niu,H., Miller,A.S. and Sung,P. (2015) Biochemical mechanism of DSB end resection and its regulation. *DNA Repair (Amst)*. **32**, 66–74.
17. Cejka,P. (2015) DNA End resection: nucleases team up with the right partners to initiate homologous recombination. *J. Biol. Chem.*, **290**, 22931–22938.
18. Paull,T.T. (2010) Making the best of the loose ends: Mre11/Rad50 complexes and Sae2 promote DNA double-strand break resection. *DNA Repair (Amst).*, **9**, 1283–1291.

19. Symington,L.S. (2016) Mechanism and regulation of DNA end resection in eukaryotes. *Crit. Rev. Biochem. Mol. Biol.*, **51**, 195–212.
20. You,Z. and Bailis,J.M. (2010) DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. *Trends Cell Biol.*, **20**, 402–409.
21. Huertas,P. (2010) DNA resection in eukaryotes: deciding how to fix the break. *Nat. Struct. Mol. Biol.*, **17**, 11–16.
22. Bernstein,K.A. and Rothstein,R. (2009) At loose ends: resecting a double-strand break. *Cell*, **137**, 807–810.
23. Abraham,R.T. (2001) Cell cycle checkpoint signaling through the ATM an ATR kinases. *Genes Dev.*, **15**, 2177–2196.
24. Shiloh,Y. and Ziv,Y. (2013) The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat. Rev. Mol. Cell Biol.*, **14**, 197–210.
25. Cimprich,K.A. and Cortez,D. (2008) ATR: an essential regulator of genome integrity. *Nat. Rev. Mol. Cell Biol.*, **9**, 616–627.
26. You,Z., Bailis,J.M., Johnson,S.A., Dilworth,S.M. and Hunter,T. (2007) Rapid activation of ATM on DNA flanking double-strand breaks. *Nat. Cell Biol.*, **9**, 1311–1318.
27. Paull,T.T. (2015) Mechanisms of ATM activation. *Annu. Rev. Biochem.*, **84**, 711–738.
28. Zou,L. and Elledge,S.J. (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*, **300**, 1542–1548.
29. Shiotani,B. and Zou,L. (2009) Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Mol. Cell*, **33**, 547–558.
30. Clerici,M., Trovesi,C., Galbiati,A., Lucchini,G. and Longhese,M.P. (2014) Mec1/ATR regulates the generation of single-stranded DNA that attenuates Tel1/ATM signaling at DNA ends. *EMBO J.*, **33**, 198–216.
31. You,Z., Shi,L.Z., Zhu,Q., Wu,P., Zhang,Y.W., Basilio,A., Tonnu,N., Verma,I.M., Berns,M.W. and Hunter,T. (2009) CtIP links DNA double-strand break sensing to resection. *Mol. Cell*, **36**, 954–969.
32. Mimitou,E.P. and Symington,L.S. (2009) DNA end resection: many nucleases make light work. *DNA Repair (Amst).*, **8**, 983–995.
33. Mimitou,E.P. and Symington,L.S. (2008) Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature*, **455**, 770–774.
34. Zhu,Z., Chung,W.H., Shim,E.Y., Lee,S.E. and Ira,G. (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell*, **134**, 981–994.
35. Garcia,V., Phelps,S.E.L., Gray,S. and Neale,M.J. (2011) Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. *Nature*, **479**, 241–244.
36. Limbo,O., Chahwan,C., Yamada,Y., de Bruin,R.A.M., Wittenberg,C. and Russell,P. (2007) Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. *Mol. Cell*, **28**, 134–146.
37. Takeda,S., Nakamura,K., Taniguchi,Y. and Paull,T.T. (2007) Ctp1/CtIP and the MRN complex collaborate in the initial steps of homologous recombination. *Mol. Cell*, **28**, 351–352.
38. Sartori,A.A., Lukas,C., Coates,J., Mistrik,M., Fu,S., Bartek,J., Baer,R., Lukas,J. and Jackson,S.P. (2007) Human CtIP promotes DNA end resection. *Nature*, **450**, 509–514.
39. Chin,G.M. and Villeneuve,A.M. (2001) C.elegans mre-11 is required for meiotic recombination and DNA repair but is dispensable for the meiotic G2 DNA damage checkpoint. *Genes Dev.*, **15**, 522–534.
40. Penkner,A., Portik-Dobos,Z., Tang,L., Schnabel,R., Novatchkova,M., Jantsch,V. and Loidl,J. (2007) A conserved function for a Caenorhabditis elegans Com1/Sae2/CtIP protein homolog in meiotic recombination. *EMBO J.*, **26**, 5071–5082.
41. Cannavo,E. and Cejka,P. (2014) Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. *Nature*, **514**, 122–125.
42. Anand,R., Ranjha,L., Cannavo,E. and Cejka,P. (2016) Phosphorylated CtIP functions as a co-factor of the MRE11-RAD50-NBS1 endonuclease in DNA end resection. *Mol. Cell*, **64**, 940–950.
43. Makharashvili,N., Tubbs,A.T., Yang,S.H., Wang,H., Barton,O., Zhou,Y., Deshpande,R.A., Lee,J.H., Lobrich,M., Sleckman,B.P. *et al.* (2014) Catalytic and noncatalytic roles of the CtIP endonuclease in double-strand break end resection. *Mol. Cell*, **54**, 1022–1033.
44. Wang,H., Li,Y., Truong,L.N., Shi,L.Z., Hwang,P.Y.-H., He,J., Do,J., Cho,M.J., Li,H., Negrete,A. *et al.* (2014) CtIP maintains stability at common fragile sites and inverted repeats by end resection-independent endonuclease activity. *Mol. Cell*, **54**, 1012–1021.
45. Lengsfeld,B.M., Rattray,A.J., Bhaskara,V., Ghirlando,R. and Paull,T.T. (2007) Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. *Mol. Cell*, **28**, 638–651.
46. Shibata,A., Moiani,D., Arvai,A.S., Perry,J., Harding,S.M., Genois,M.-M., Maity,R., van Rossum-Fikkert,S., Kertokalio,A., Romoli,F. *et al.* (2014) DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. *Mol. Cell*, **53**, 7–18.
47. Broderick,R., Nieminuszczy,J., Baddock,H.T., Deshpande,R.A., Gileadi,O., Paull,T.T., Mchugh,P.J. and Niedzwiedz,W. (2016) EXD2 promotes homologous recombination by facilitating DNA end resection. *Nat. Cell Biol.*, **18**, 271–280.
48. Nicolette,M.L., Lee,K., Guo,Z., Rani,M., Chow,J.M., Lee,S.E. and Paull,T.T. (2010) Mre11–Rad50–Xrs2 and Sae2 promote 5′ strand resection of DNA double-strand breaks. *Nat. Struct. Mol. Biol.*, **17**, 1478–1485.
49. Gravel,S., Chapman,J.R., Magill,C. and Jackson,S.P. (2008) DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes Dev.*, **22**, 2767–2772.
50. Nimonkar,A. V., Ozsoy,A.Z., Genschel,J., Modrich,P. and Kowalczykowski,S.C. (2008) Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 16906–16911.
51. Nimonkar,A. V, Genschel,J., Kinoshita,E., Polaczek,P., Campbell,J.L., Wyman,C., Modrich,P. and Kowalczykowski,S.C. (2011) BLM – DNA2 – RPA – MRN and EXO1 – BLM – RPA – MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev.*, **25**, 350–362.
52. Chen,X., Paudyal,S.C., Chin,R.-I. and You,Z. (2013) PCNA promotes processive DNA end resection by Exo1. *Nucleic Acids Res.*, **41**, 9325–9338.
53. Ngo,G.H.P., Balakrishnan,L., Dubarry,M., Campbell,J.L. and Lydall,D. (2014) The 9-1-1 checkpoint clamp stimulates DNA resection by Dna2-Sgs1 and Exo1. *Nucleic Acids Res.*, **42**, 10516–10528.
54. Ngo,G.H.P. and Lydall,D. (2015) The 9-1-1 checkpoint clamp coordinates resection at DNA double strand breaks. *Nucleic Acids Res.*, **43**, 5017–5032.
55. Sturzenegger,A., Burdova,K., Kanagaraj,R., Levikova,M., Pinto,C., Cejka,P. and Janscak,P. (2014) DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA-end resection in human cells. *J. Biol. Chem.*, **289**, 27314–27326.
56. Daley,J.M., Chiba,T., Xue,X., Niu,H. and Sung,P. (2014) Multifaceted role of the Topo IIIα-RMI1-RMI2 complex and DNA2 in the BLM-dependent pathway of DNA break end resection. *Nucleic Acids Res.*, **42**, 11083–11091.
57. Cejka,P., Cannavo,E., Polaczek,P., Masuda-Sasa,T., Pokharel,S., Campbell,J.L. and Kowalczykowski,S.C. (2010) DNA end resection by Dna2–Sgs1–RPA and its stimulation by Top3–Rmi1 and Mre11–Rad50–Xrs2. *Nature*, **467**, 112–116.
58. Niu,H., Chung,W.-H., Zhu,Z., Kwon,Y., Zhao,W., Chi,P., Prakash,R., Seong,C., Liu,D., Lu,L. *et al.* (2010) Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae. *Nature*, **467**, 108–111.
59. Liao,S., Toczylowski,T. and Yan,H. (2008) Identification of the Xenopus DNA2 protein as a major nuclease for the 5′→3′ strand-specific processing of DNA ends. *Nucleic Acids Res.*, **36**, 6091–6100.
60. Yan,H., Toczylowski,T., McCane,J., Chen,C. and Liao,S. (2011) Replication protein A promotes 5′-3′ end processing during homology-dependent DNA double-strand break repair. *J. Cell Biol.*, **192**, 251–261.
61. Tammaro,M., Liao,S., McCane,J. and Yan,H. (2015) The N-terminus of RPA large subunit and its spatial position are important for the 5′→3′ resection of DNA double-strand breaks. *Nucleic Acids Res.*, **43**, 8790–8800.

62. Tammaro,M., Liao,S., Beeharry,N. and Yan,H. (2016) DNA double-strand breaks with 5 adducts are efficiently channeled to the DNA2-mediated resection pathway. *Nucleic Acids Res.*, **44**, 221–231.
63. Toczylowski,T. and Yan,H. (2006) Mechanistic analysis of a DNA end processing pathway mediated by the Xenopus Werner syndrome protein. *J. Biol. Chem.*, **281**, 33198–33205.
64. Zhang,H., Hua,Y., Li,R. and Kong,D. (2016) Cdc24 is essential for long-range end resection in the repair of double-stranded DNA breaks. *J. Biol. Chem.*, **291**, 24961–24973.
65. Langerak,P., Mejia-Ramirez,E., Limbo,O. and Russell,P. (2011) Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. *PLoS Genet.*, **7**, e1002271.
66. Neale,M.J., Pan,J. and Keeney,S. (2005) Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature*, **436**, 1053–1057.
67. Hartsuiker,E., Mizuno,K., Molnar,M., Kohli,J., Ohta,K. and Carr,A.M. (2009) Ctp1CtIP and Rad32Mre11 nuclease activity are required for Rec12Spo11 removal, but Rec12Spo11 removal is dispensable for other MRN-dependent meiotic functions. *Mol. Cell. Biol.*, **29**, 1671–1681.
68. Hartsuiker,E., Neale,M.J. and Carr,A.M. (2009) Distinct requirements for the Rad32Mre11 nuclease and Ctp1CtIP in the removal of covalently bound topoisomerase I and II from DNA. *Mol. Cell*, **33**, 117–123.
69. Aparicio,T., Baer,R., Gottesman,M. and Gautier,J. (2016) MRN, CtIP, and BRCA1 mediate repair of topoisomerase II–DNA adducts. *J. Cell Biol.*, **212**, 399–408.
70. Deshpande,R.A., Lee,J.H., Arora,S. and Paull,T.T. (2016) Nbs1 converts the human Mre11/Rad50 nuclease complex into an endo/exonuclease machine specific for protein-DNA adducts. *Mol. Cell*, **64**, 593–606.
71. Hoa,N.N., Shimizu,T., Zhou,Z.W., Wang,Z.Q., Deshpande,R.A., Paull,T.T., Akter,S., Tsuda,M., Furuta,R., Tsusui,K. *et al.* (2016) Mre11 is essential for the removal of lethal topoisomerase 2 covalent cleavage complexes. *Mol. Cell*, **64**, 580–592.
72. Rothenberg,M., Kohli,J. and Ludin,K. (2009) Ctp1 and the MRN-complex are required for endonucleolytic Rec12 removal with release of a single class of oligonucleotides in fission yeast. *PLoS Genet.*, **5**, e1000722.
73. Nakamura,K., Kogame,T., Oshiumi,H., Shinohara,A., Sumitomo,Y., Agama,K., Pommier,Y., Tsutsui,K.M., Tsutsui,K., Hartsuiker,E. *et al.* (2010) Collaborative action of Brca1 and CtIP in elimination of covalent modifications from double-strand breaks to facilitate subsequent break repair. *PLoS Genet.*, **6**, e1000828.
74. Deng,C., Brown,J.A., You,D. and Brown,J.M. (2005) Multiple endonucleases function to repair covalent topoisomerase I complexes in Saccharomyces cerevisiae. *Genetics*, **170**, 591–600.
75. Cannavo,E., Cejka,P. and Kowalczykowski,S.C. (2013) Relationship of DNA degradation by Saccharomyces cerevisiae Exonuclease 1 and its stimulation by RPA and Mre11-Rad50-Xrs2 to DNA end resection. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, E1661–E1668.
76. Moreau,S., Ferguson,J.R. and Symington,L.S. (1999) The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance. *Mol. Cell. Biol.*, **19**, 556–566.
77. Llorente,B. and Symington,L.S. (2004) The Mre11 nuclease is not required for 5′ to 3′ resection at multiple HO-induced double-strand breaks. *Mol. Cell. Biol.*, **24**, 9682–9694.
78. Ivanov,E.L., Sugawara,N., White,C.I., Fabre,F. and Haber,J.E. (1994) Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **14**, 3414–3425.
79. Clerici,M., Mantiero,D., Lucchini,G. and Longhese,M.P. (2005) The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends. *J. Biol. Chem.*, **280**, 38631–38638.
80. Liao,S., Tammaro,M. and Yan,H. (2016) The structure of ends determines the pathway choice and Mre11 nuclease dependency of DNA double-strand break repair. *Nucleic Acids Res.*, **44**, 5689–5701.
81. Walter,J., Sun,L. and Newport,J. (1998) Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell*, **1**, 519–529.
82. Lupardus,P.J., Van,C. and Cimprich,K.A. (2007) Analyzing the ATR-mediated checkpoint using Xenopus egg extracts. *Methods*, **41**, 222–231.
83. Srinivasan,S.V. and Gautier,J. (2011) Study of cell cycle checkpoints using Xenopus cell-free extracts. *Methods Mol. Biol.*, **782**, 119–158.
84. Garner,E. and Costanzo,V. (2009) Studying the DNA damage response using in vitro model systems. *DNA Repair (Amst).*, **8**, 1025–1037.
85. Cheruiyot,A., Paudyal,S.C., Kim,I., Sparks,M., Ellenberger,T., Piwnica-Worms,H. and You,Z. (2015) Poly (ADP-ribose) -binding promotes Exo1 damage recruitment and suppresses its nuclease activities. *DNA Repair (Amst).*, **35**, 106–115.
86. Chen,X., Kim,I., Honaker,Y., Paudyal,S.C., Koh,W.K., Sparks,M., Li,S., Piwnica-Worms,H., Ellenberger,T. and You,Z. (2015) 14-3-3 Proteins restrain the Exo1 nuclease to prevent overresection. *J. Biol. Chem.*, **290**, 12300–12312.
87. You,Z., Chahwan,C., Bailis,J., Hunter,T. and Russell,P. (2005) ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol. Cell. Biol.*, **25**, 5363–5379.
88. Liao,S., Guay,C., Toczylowski,T. and Yan,H. (2012) Analysis of MRE11's function in the 5′→3′ processing of DNA double-strand breaks. *Nucleic Acids Res.*, **40**, 4496–4506.
89. Peterson,S.E., Li,Y., Chait,B.T., Gottesman,M.E., Baer,R. and Gautier,J. (2011) Cdk1 uncouples CtIP-dependent resection and Rad51 filament formation during M-phase double-strand break repair. *J. Cell Biol.*, **194**, 705–720.
90. Peterson,S.E., Li,Y., Wu-Baer,F., Chait,B.T., Baer,R., Yan,H., Gottesman,M.E. and Gautier,J. (2013) Activation of DSB processing requires phosphorylation of CtIP by ATR. *Mol. Cell*, **49**, 657–667.
91. Murphy,C.I., Piwnica-Worms,H., Grünwald,S., Romanow,W.G., Francis,N. and Fan,H.-Y. (2004) Expression and purification of recombinant proteins using the Baculovirus system. *Curr. Protoc. Mol. Biol*. **16**, doi:10.1002/0471142727.mb1611s65.
92. Lee,B.I. and Wilson,D.M. (1999) The RAD2 domain of human exonuclease 1 exhibits 5′ to 3′ exonuclease and flap structure-specific endonuclease activities. *J. Biol. Chem.*, **274**, 37763–37769.
93. Budd,M.E. and Campbell,J.L. (1995) A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 7642–7646.
94. Imamura,O. and Campbell,J.L. (2003) The human Bloom syndrome gene suppresses the DNA replication and repair defects of yeast dna2 mutants. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 8193–8198.
95. Liu,Q., Choe,W. and Campbell,J.L. (2000) Identification of the Xenopus laevis Homolog of Saccharomyces cerevisiae DNA2 and Its Role in DNA Replication. *J. Biol. Chem.*, **275**, 1615–1624.
96. Masuda-sasa,T., Imamura,O. and Campbell,J.L. (2006) Biochemical analysis of human Dna2. *Nucleic Acids Res.*, **34**, 1865–1875.
97. Balakrishnan,L., Polaczek,P., Pokharel,S., Campbell,J.L. and Bambara,R.A. (2010) Dna2 exhibits a unique strand end-dependent helicase. *J. Biol. Chem.*, **285**, 38861–38868.
98. Pinto,C., Kasaciunaite,K., Seidel,R. and Cejka,P. (2016) Human DNA2 possesses a cryptic DNA unwinding activity that functionally integrates with BLM or WRN helicases. *Elife*, **5**, e18574.
99. Levikova,M., Klaue,D., Seidel,R. and Cejka,P. (2013) Nuclease activity of Saccharomyces cerevisiae Dna2 inhibits its potent DNA helicase activity. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, E1992–E2001.
100. Lin,W., Sampathi,S., Dai,H., Liu,C., Zhou,M., Hu,J., Huang,Q., Campbell,J., Shin-Ya,K., Zheng,L. *et al.* (2013) Mammalian DNA2 helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity. *EMBO J.*, **32**, 1425–1439.
101. Dupré,A., Boyer-Chatenet,L., Sattler,R.M., Modi,A.P., Lee,J.-H., Nicolette,M.L., Kopelovich,L., Jasin,M., Baer,R., Paull,T.T. *et al.* (2008) A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nat. Chem. Biol.*, **4**, 119–125.
102. Shim,E.Y., Chung,W.-H., Nicolette,M.L., Zhang,Y., Davis,M., Zhu,Z., Paull,T.T., Ira,G. and Lee,S.E. (2010) Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. *EMBO J.*, **29**, 3370–3380.
103. Chen,H., Lisby,M. and Symington,L. (2013) RPA coordinates DNA end resection and prevents formation of DNA hairpins. *Mol. Cell*, **50**, 589–600.
104. Deng,S.K., Gibb,B., de Almeida,M.J., Greene,E.C. and Symington,L.S. (2014) RPA antagonizes microhomology-mediated

repair of DNA double-strand breaks. *Nat. Struct. Mol. Biol.*, **21**, 405–412.
105. Zhou,C., Pourmal,S. and Pavletich,N.P. (2015) Dna2 nuclease-helicase structure, mechanism and regulation by Rpa. *Elife*, **4**, e09832.
106. Ryu,J. and Koo,H. (2017) The Caenorhabditis elegans WRN helicase promotes double-strand DNA break repair by mediating end resection and checkpoint activation. *FEBS Lett.*, **591**, 2155–2166.
107. Lu,H., Shamanna,R.A., Keijzers,G., Anand,R., Rasmussen,L.J., Cejka,P., Croteau,D.L. and Bohr,V.A. (2016) RECQL4 promotes DNA end resection in repair of DNA double-strand breaks. *Cell Rep.*, **16**, 161–173.
108. Kim,J., Kim,H., Ryu,G., Kim,D., Hurwitz,J. and Seo,Y. (2006) Isolation of human Dna2 endonuclease and characterization of its enzymatic properties. *Nucleic Acids Res.*, **34**, 1854–1864.
109. Duxin,J.P., Moore,H.R., Sidorova,J., Karanja,K., Honaker,Y., Dao,B., Piwnica-Worms,H., Campbell,J.L., Monnat,R.J. and Stewart,S.A. (2012) Okazaki fragment processing-independent role for human Dna2 enzyme during DNA replication. *J. Biol. Chem.*, **287**, 21980–21991.
110. Miller,A.S., Daley,J.M., Pham,N.T., Niu,H., Xue,X., Ira,G. and Sung,P. (2017) A novel role of the Dna2 translocase function in DNA break resection. *Genes Dev.*, **31**, 503–510.
111. Levikova,M., Pinto,C. and Cejka,P. (2017) The motor activity of DNA2 functions as an ssDNA translocase to promote DNA end resection. *Genes Dev.*, **31**, 493–502.