

Analysis of MRE11's function in the 5'→3' processing of DNA double-strand breaks

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ABSTRACT

The resection of DNA double-strand breaks (DSBs) into 3' single-strand tails is the initiating step of homology-dependent repair pathways. A key player in this process is the MRE11-RAD50-NBS1 complex, but its contribution to and mechanistic role in resection are not well understood. In this study, we took advantage of the *Xenopus* egg extract system to address these questions. We found that depletion of MRE11 caused a dramatic inhibition of 5'-resection, even for the first nucleotide at the 5'-end. Depletion of *Xenopus* CtIP also inhibited 5'-strand resection, but this inhibition could be alleviated by excess MRN. Both MRE11 and CtIP could be bypassed by a DNA that carried a 3'-ss-tail. Finally, using purified proteins, we found that MRN could stimulate both the WRN-DNA2-RPA pathway and the EXO1 pathway of resection. These findings provide important insights into the function of MRE11 in 5'-strand resection.

INTRODUCTION

Among the numerous DNA lesions that might occur naturally or induced by genotoxic agents, DNA double-strand breaks (DSBs) are among the most deleterious to cells. If unrepaired or improperly repaired, DSBs would lead to chromosome deletions, translocations, duplications and ultimately premature cell death or oncogenic transformation. In eukaryotes, there are three major types of pathways for repairing DSBs: non-homologous end joining (NHEJ), homologous recombination (HR) and single-strand annealing (SSA) (1). NHEJ is accomplished by directly ligating the ends, usually after some minor polishing to remove damaged nucleotides or fill-in the 5'-protruding ends. HR repairs DSBs by invading a homologous sequence to copy the missing information. If a DSB occurs between two direct repeat sequences, it can also be repaired by SSA and the final product retains effectively only one of the two repeat sequences. HR and

SSA are both dependent on sequence homology and are thus also collectively referred to as homology-dependent DSB repair.

The crucial event that determines the choice of repair pathways is the initial processing of DNA ends. NHEJ is associated with limited processing, but HR and SSA require extensive resection of the 5'-strand to generate the 3'-ss-tail. In HR, the 3'-ss-tail invades a homologous sequence, whereas in SSA the two 3'-ss-tails from each side of the break anneal with each other. The molecular pathways responsible for 5'-strand resection in various eukaryotic systems are beginning to be elucidated. Studies in budding and fission yeasts, extracts derived from *Xenopus* eggs, and human cells have led to the identification of two major pathways for the resection of 5'-strands. One pathway is mediated by the combined actions of a RecQ-type DNA helicase (Sgs1, BLM or WRN) and the DNA2 nuclease (2–6). DNA ends are first unwound by the helicase and the resulting 5'-ss-tail is then degraded by the 5'→3'-nuclease activity of DNA2, leaving the 3'-tail as the product (2). Both steps are dependent on the eukaryotic ss-DNA-binding protein RPA, which interacts physically with both the helicase and the nuclease and stimulates their activities (7). The other pathway for 5'-strand processing is mediated by EXO1, a highly conserved member of the RADII nuclease family. Inactivation of EXO1 in *Saccharomyces cerevisiae*, *Xenopus* and human cells all led to a reduction of 5'-resection (3,5,6,8). Its mechanism of action is distinct from that of DNA2. Instead of acting on ss-DNA, EXO1 uses its 5'→3'-ds-DNA exonuclease activity to directly degrade ds-DNA (8).

The helicases and exonucleases described above are not the only players in 5'-strand resection. Studies in *S. cerevisiae* showed that *exo1 sgs1* double mutants could still carry out limited 5'-resection of a few hundred nucleotides adjacent to the break (5,6). The MRE11-RAD50-XRS complex (MRX) and SAE2 appear to be responsible for the initial limited processing of the 5'-strand. Inactivation of any of these genes causes accumulation of apparently unresected DSBs, but a large fraction of ends escape the inhibition and are resected at

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the normal rate. On average, inactivating MRX caused only a 2-fold decrease in resection in actively dividing cells (9). This raised an important question as to why MRX is not required for all ends. There is strong evidence that 'dirty' ends cross-linked with proteins like SPO11 might require MRX for the initial cleavage to remove the protein adduct. However, the ends generated by HO endonuclease, which are presumably clean, can still be either dependent on or independent of MRX (5,6). Another important question is how MRX acts with SAE2 to initiate a limited resection before SGS1-DNA2 or EXO1 take over for more extensive resection. It is unclear how the enzymatic activities of MRX and SAE2 themselves can accomplish the initial resection. MRE11 displays ss-DNA endonuclease activity and 3'→5'-ds-DNA exonuclease activity, while SAE2 displays DNA hairpin endonuclease activity (10–12). None of these activities is directly compatible with the 5'→3'-directionality of strand resection at DSBs. Finally, while *in vivo* data suggest that MRX and SAE2 are both important for 5'-strand resection, two studies with the purified end resection proteins found that SAE2 was completely dispensable for end resection (13,14). Deciphering the functional relationship between MRX and SAE2 is crucial to the understanding how they promote 5'-strand resection.

The functions of MRX and SAE2 appear to be highly conserved. Their vertebrate homologues, MRN (MRE11-RAD450-NBS1) and CtIP respectively, are also important for strand resection in mammalian cells and *Xenopus* egg extracts (15–19). Of particular importance to the understanding of MRE11's role in 5'-strand resection is the potential application of the *Xenopus* system. We have previously successfully reconstituted 5'→3'-strand processing in nucleoplasmic extracts (NPE), which is derived from nuclei formed in crude interphase egg extracts (2). Using this system, we discovered that one major pathway consists of two coupled reactions: an ATP-dependent unwinding of ends and the degradation of the resulting 5'-ss-tails, leaving the 3'-ss-tail as the final product. The major DNA helicase for end unwinding is the *Xenopus* Werner syndrome protein and the major 5'→3'-ss-DNA exonuclease is the *Xenopus* DNA2 protein (2,4). The eukaryotic ss-DNA-binding protein RPA facilitates 5'-strand resection by stimulating the helicase activity of WRN and the 5'→3'-ss-DNA exonuclease activity of DNA2 via direct physical interactions (7). More recently, we established that cytosol, the membrane-free fraction of crude interphase *Xenopus* egg extracts after ultracentrifugation, is also capable of bona fide 5'→3'-end processing (8). Compared to NPE, cytosol is far easier to prepare and to deplete proteins of interest. Using this more convenient system, we found that in addition to the WRN-DNA2-RPA pathway, which is also active in cytosol, *Xenopus* EXO1 constitutes a parallel pathway for 5'→3'-end processing. But unlike DNA2, EXO1 acts directly on ds-DNA to degrade it in the 5'→3' direction. Together, these results suggest strongly that the end processing pathways are highly conserved in the *Xenopus* system.

In this study, we took advantage of the *Xenopus* egg extract system to investigate the mechanistic role of MRE11 in 5'-strand resection. We found that immunodepletion of MRE11 caused a dramatic block to 5'-resection of linear DNA. The ends were generated by restriction enzyme digestion, indicating that MRE11 is required for the resection of even 'clean' ends. The block was from the very beginning and even the first nucleotide at the 5'-end was not efficiently resected in the absence of MRE11. Depletion of *Xenopus* CtIP also inhibited 5'-strand resection, but this inhibition could be bypassed by increased amount of MRN, suggesting that CtIP's role in resection is to stimulate the activity of MRN. In addition, the requirements for MRE11 and CtIP could be bypassed by a DNA that carried a 3'-ss-tail, suggesting that they are required only in the initiation step but not the extension step of resection. Finally, using purified proteins, we found that MRN could stimulate both the WRN-DNA2-RPA pathway and the EXO1 pathway of resection. These results provide important insights into the function and mechanism of action of MRE11 in DSB repair.

MATERIALS AND METHODS

Cytosol preparation

Membrane-free cytosol of interphase *Xenopus* eggs and NPE were prepared following the published protocols (20).

Antibody production and purification

The full-length *Xenopus* MRE11 was expressed as a His-tagged fusion protein in bacterial BL21(DE3) cells using a construct as published (21). The resulting inclusion body was purified and the MRE11 protein was further separated on a SDS-PAGE gel. The band corresponding to the full-length His-MRE11 was isolated and injected into two rabbits. Anti-*Xenopus* CtIP antibodies and anti-*Xenopus* RAD50 antibodies were prepared in a similar way. Other antibodies used in the study, rabbit anti-*Xenopus* EXO1 and rabbit anti-*Xenopus* WRN, have been published (4,22).

Expression and purification of recombinant MRN

Full-length *Xenopus* MRE11 was purified as described (21). Full-length *Xenopus* NBS1 and the N-terminus of *Xenopus* RAD50 were purchased from Open Biosystems. The C-terminus of *Xenopus* RAD50 was isolated by PCR from a *Xenopus* oocyte cDNA library and then cloned downstream of the N-terminus to make the full-length RAD50. MRE11 and RAD50 were cloned into a modified pBAC-Dual vector and NBS1 was subcloned into the pBAC vector. Viruses that contain MRE11 and RAD50 or NBS were prepared according to the standard protocol. Sf9 cells were co-infected with both viruses and collected after 72 h. Cell pellets were resuspended in ice-cold lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM NaCl and 1% NP-40) plus a cocktail of protease inhibitors (Sigma-Aldrich, MO, USA) and then lysed by a

Dounce homogenizer. The nuclei were separated from the cytoplasm by centrifugation (JS13.1 rotor, at 3000 rpm for 10 min in 4°C). The nuclear pellets were resuspended in ice-cold extraction buffer (25 mM Tris-HCl, pH 8.0 and 500 mM NaCl) plus a cocktail of protease inhibitors. The nuclear proteins were then extracted by incubating on ice for 20 min. The nuclear extract was further purified by FPLC columns. The nuclear extract was first diluted to lower the NaCl concentration to 100 mM and then loaded onto a 1 ml HiTrap Q-column. The column was eluted with a linear gradient of NaCl from 100 to 500 mM. The peak of xMRN was eluted in 240–320 mM NaCl. The pooled Q-column fractions were again diluted to lower the NaCl concentration to 100 mM and loaded onto a 1 ml HiTrap S-column. The peak of xMRN was eluted in 280–340 mM NaCl. The pooled peak of xMRN was further purified by a 1 ml HiTrap Heparin column with a linear gradient of NaCl from 100 to 600 mM. The majority of xMRN was eluted off the Heparin column in 400–475 mM NaCl. The Heparin fractions of xMRN were pooled and concentrated down to 1 ml and last purified by a Superdex 200 (10/30) gel-filtration column. The purest xMRN was eluted in the ninth milliliter (near the void volume) and used for complementation and biochemical experiments.

Immunodepletion

To deplete MRE11, cytosol {40 μ l + 20 μ l ELB [10 mM HEPES (pH 7.5), 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 1 mM DTT]} was incubated with 20 μ l Protein A Sepharose beads pre-coated with 80 μ l of the rabbit anti-MRE11 serum or pre-serum at 4°C for 1.5 h. The procedure was repeated once and the depleted cytosol was saved as 5 μ l aliquots at -80°C. Depletions of CtIP, WRN and EXO1 were carried following the same method using the corresponding antibodies.

5'-strand resection assays

The 3'-³²P-labeled substrate for resection assays was prepared by digesting plasmid pBLP with BamHI and filling the ends with dGTP, dTTP, ddCTP and ³²P dATP. The 5'-³²P-labeled substrate was similarly prepared except for that cold dATP replaced ³²P dATP in the fill-in reaction and the 5'-end was phosphorylated by γ -³²P ATP with T4 polynucleotide kinase (NEB, MA, USA). For DNA with a 3'-ss-tail, the 3'-labeled DNA (20 ng/ μ l) was treated with lambda exonuclease (0.07 unit/ μ l; NEB, MA, USA) in ELB buffer at 4°C for 60 s. All substrates were purified by Qiagen nucleotide removal kit before use. A typical assay contained: 5 μ l depleted cytosol, 0.5 μ l 10 \times ATP mix (20 mM ATP/200 mM phosphocreatine/0.5 mg/ml creatine kinase/50 mM DTT), 0.375 μ l DNA (20 ng/ μ l), 0.15 μ l 10 mM ddCTP, 1.5 μ l appropriate protein (for complementation) or ELB buffer (total volume = ca. 7.5 μ l). The reactions were incubated at room temperature (22–25°C), samples were taken at the indicated times and mixed with equal volume of 2% SDS/25 mM EDTA. At the end, samples were brought up to 10 μ l with H₂O and 1 μ l proteinase K (10 mg/ml). After incubation at room temperature for 2 h, samples were

separated on 1% TAE/agarose gels. DNA was detected by staining with SYBR Gold (Invitrogen, CA, USA) and by exposure to Phosphorimager (Fuji) or film after drying.

Reconstitution of MRN-WRN-DNA2-RPA and MRN-EXO1 pathways with purified proteins

For the reconstitution of the MRN-WRN-DNA2-RPA pathway, the reactions contained ~16 ng/ μ l MRN, 2 ng/ μ l WRN, 2 ng/ μ l DNA2, 6 ng/ μ l RPA and 1 \times ATP regeneration system. For the reconstitution of the MRN-EXO1 pathway, reactions contained ca. 16 ng/ μ l MRN and 0.2 ng/ μ l EXO1. The salt concentrations in the reactions were equivalent to ca. 100 mM NaCl. Samples were taken at the indicated times, stopped with 1% SDS and 12.5 mM EDTA and analyzed similarly to that described for the resection assays above.

Protein interaction experiments

For the interactions between MRN and DNA2 or RPA, 1 μ l Protein A magnetic beads (Invitrogen, CA, USA) pre-coated with anti-MRE11 antibodies were incubated with ca. 16 ng/ μ l MRN, alone or combined with 6 ng/ μ l RPA or 2 ng/ μ l DNA2 and 1 μ g/ μ l BSA in 5 μ l reactions (salt concentration equivalent to ca. 100 mM NaCl). After 1 h at 4°C, the beads were washed twice with 20 μ l A100+0.005% NP40. The supernatant and beads fractions were separated on 4–12% SDS-PAGE (Invitrogen, CA, USA), transferred to a PVDF membrane and probed for RPA, DNA2 and RAD50 by Western blot. Detection was achieved with SuperSignal Chemiluminescence (Pierce Chemical Co. IL, USA). For the interaction between MRN and EXO1, 1 μ l Streptavidin magnetic beads (Invitrogen, CA, USA) coated with 1.5 kb DNA at one end (20 ng/ μ l beads) were incubated with 16 ng/ μ l MRN, 1 ng/ μ l EXO1, alone or together in the presence of 0.5 μ g/ μ l BSA (total volume = 10 μ l; salt concentration equivalent to ca. 100 mM NaCl+15 mM EDTA). After 30 min at 4°C, the beads were washed twice with 20 μ l A100+0.005% NP40. The supernatant and beads fractions were analyzed for EXO1 and RAD50 by Western blot similarly to that described above.

RESULTS

MRE11 is required for 5'→3' processing of DNA ends in *Xenopus* egg extracts

To study the role of MRE11 in 5'-strand resection, we first prepared antibodies against its *Xenopus* homologue. As shown in Figure 1A, the anti-MRE11 antibodies, but not the pre-immune, detected a band corresponding to the expected size of MRE11 (81 kd) in cytosol. The same size band was also detected in NPE, which is consistent with the expected nuclear localization of MRN. The antibody was coated onto Protein A Sepharose beads and the beads were then used to deplete xMRE11 from cytosol. As shown in Figure 1B, MRE11 could be depleted to a level below detection (>99% depletion). To assay for the effect on 5'-strand resection, the MRE11-depleted extract and mock-depleted extract were

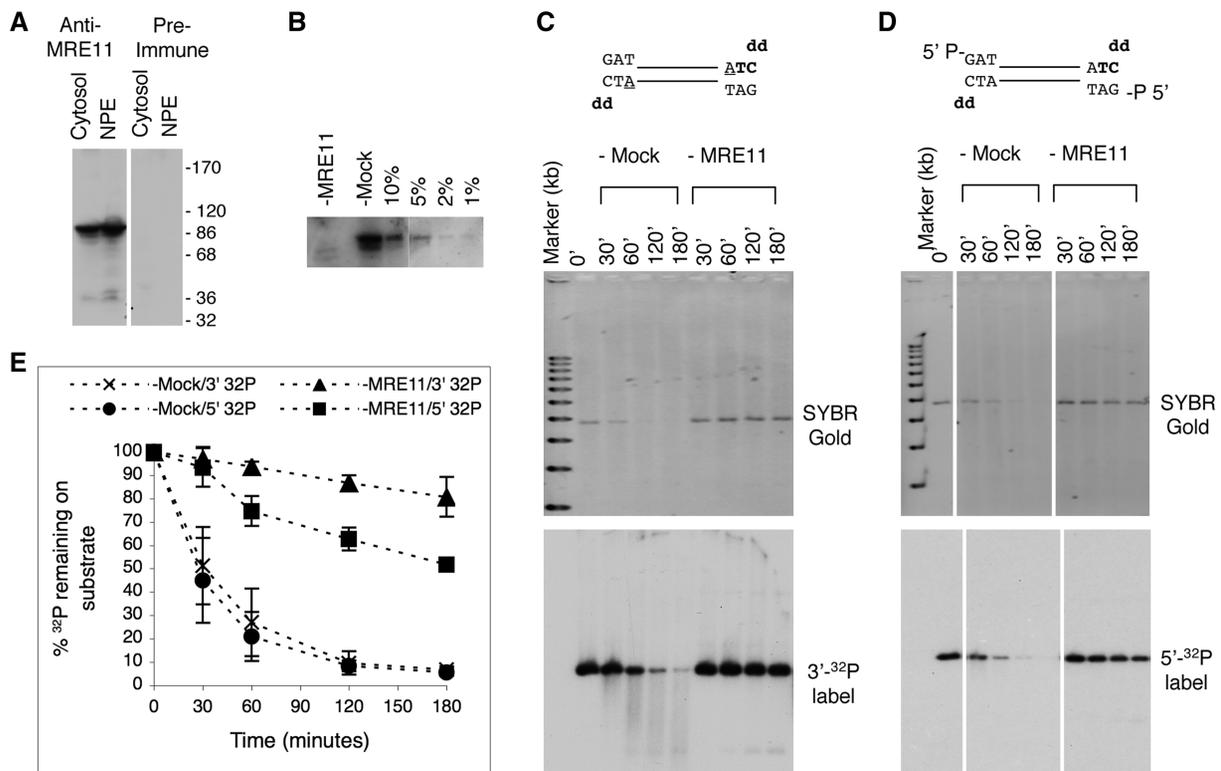


Figure 1. Depletion of MRE11 blocks 5'-strand resection. (A) Detection of MRE11 in cytosol and NPE by Western blot with anti-*Xenopus* MRE11 antibodies. (B) Efficiency of MRE11 depletion from cytosol. The standards for quantitation were cytosol loaded at different amounts relative to the depleted cytosol. (C) Effect of MRE11 depletion on 5'-strand resection. 3'-³²P-labeled (at the underlined A) linear pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated in the MRE11-depleted or mock-depleted cytosol. Samples were taken at the indicated times, treated with SDS/proteinase K and separated on a 1% TAE/agarose gel. The gel was first stained with SYBR Gold and then dried for exposure to an X-ray film. (D) Effect of MRE11 depletion on the initiation of 5'-strand processing. The 5'-³²P-labeled linear pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated with the MRE11-depleted or mock-depleted cytosol. Samples taken at the indicated times were analyzed as in (C). (E) Plot of the amounts of 3'- and 5'-³²P on the remaining substrates at the indicated times after incubation in the MRE11-depleted or mock-depleted extracts. The averages and standard deviations were calculated with three sets of data.

incubated with a linear DNA with ddC-terminated ends (to block NHEJ). As shown in Figure 1C, the DNA was efficiently degraded in the mock-depleted extract, as demonstrated by both SYBR Gold staining and ³²P label at the 3'-end. The 3'-label was retained on the intermediates, consistent with the 5'→3' directionality of end processing established previously (8). In the MRE11-depleted extract, however, the DNA remained stable, with on average 81% of the 3'-³²P label still remaining on the substrate even after 3 h of incubation (Figure 1E). To further determine if there was any resection of the 5'-strand in the absence of MRE11, we also prepared a DNA with a ³²P label at the 5'-end by T4 kinase. As shown in Figure 1D, the 5'-³²P was efficiently removed after incubation in the mock-depleted cytosol. In contrast, it was very stable in the MRE11-depleted cytosol. Even after 3 h of incubation, on average 52% of the 5'-³²P was still retained on the substrate (Figure 1E).

To confirm that the observed effect of MRE11 depletion was specific rather than the result of spurious co-depletion of other factors, we attempted to complement it with the recombinant MRE11 protein. MRE11 exists in a complex with RAD50 and NBS1 and depletion of MRE11 co-depletes RAD50 and most of NBS1 (23).

We thus also cloned the *Xenopus* RAD50 and NBS1 and co-expressed them with MRE11 in insect cells. The recombinant MRN complex was purified to apparent homogeneity. The peak fraction (#9) from the final gel-filtration column (Figure 2A) was eluted at the position of ca. 500kd size, which corresponded well with the 2:2:1 stoichiometry calculated based on the staining intensities of the MRE11, RAD50 and NBS1 bands on SDS-PAGE. When this recombinant MRN complex was added to the MRE11-depleted extract, the DNA was again efficiently degraded (Figure 2B and C). Together these data showed that MRE11 is an essential component of the 5'-strand resection machinery and is required for efficiently resecting even the first nucleotide.

CtIP is also important for 5'-strand resection but can be bypassed by excess MRN

The function of MRN in 5'-strand processing is closely linked to that of CtIP. A previous study in the interphase *Xenopus* egg extract has found that depletion of CtIP inhibits the binding of RPA to chromatin, which presumably indicates the generation of ss-DNA at DSBs (18). The DNA degradation assay as shown in Figure 1 offers direct

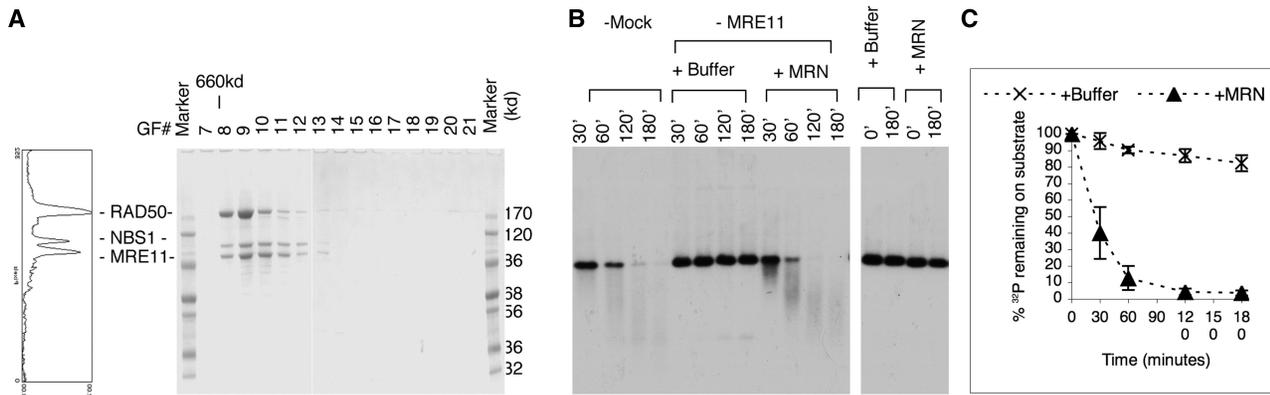


Figure 2. Complementation of MRE11 depletion by the purified MRN protein. (A) A SDS-PAGE showing the final gel-filtration fractions of the purified *Xenopus* MRN complex. The gel was stained with Coomassie blue and the staining intensity of fraction #9 was plotted. (B) Complementation of MRE11 depletion with the purified MRN complex. The 3'-³²P labeled linear pBLP DNA substrate was incubated in the MRE11-depleted cytosol supplemented with MRN or buffer. Samples taken at the indicated times were analyzed as in Figure 1C. (C) Plot of the complementation assays. The 3'-³²P on the remaining substrate DNA at the indicated times from three experiments were used to calculate the averages and standard deviations.

and nucleotide-level resolution of the fate of the 5'-strand. We thus used this assay to further define the effect of CtIP on 5'-strand resection and its relationship to MRN. Antibodies to *Xenopus* CtIP were prepared and used to deplete it from cytosol to a level below detection (<1%) (Figure 3A). When the 3'-labeled DNA was incubated in the CtIP-depleted cytosol, it was much more stable than in the mock-depleted cytosol (Figure 3B and E). Even after 3 h of incubation, most of the substrate still migrated at the original position. To further define how CtIP depletion affected 5'-strand processing, we also analyzed the fate of the 5'-labeled DNA. As shown Figure 3C, the removal of ³²P was also significantly slowed down in the CtIP-depleted cytosol when compared to in the mock-depleted cytosol. These data are consistent with the previous study but showed with a direct and high-resolution assay that CtIP is indeed important for 5'-strand resection.

We noticed that the effect of CtIP depletion on 5'-strand resection was slightly less severe than that of MRE11 depletion, as indicated by the reduction of the ³²P label and the appearance of faster migrating products. Interestingly, several reconstitution studies using purified proteins found a dependence on MRN but not on CtIP (or SAE2) (13,14,24). These observations led us to hypothesize that it might be possible to bypass CtIP's function with excess MRN. We tested this hypothesis by adding excess (approximately two times the endogenous level) MRN to the CtIP-depleted cytosol. As shown in Figure 3D and E), the addition of excess MRN caused a dramatic complementation of the CtIP-depleted cytosol. The DNA in the MRN-supplemented CtIP-depleted cytosol was now efficiently resected. The purified MRN by itself showed very little activity towards the DNA. In addition, the level of MRE11 was not affected by CtIP depletion (Figure 3A), so this rescue was not simply due to restoring the co-depleted (if any) MRN. Together, these data strongly suggest that the role of CtIP in resection is to stimulate the activity of MRN and can thus be bypassed by excess MRN.

Excess MRN cannot bypass WRN and EXO1

We next determined whether excess MRN could also bypass WRN and EXO1, which are the mechanical components responsible for the two major pathways of 5'-strand resection in the *Xenopus* extract systems. As reported previously (8), double depletion of WRN and EXO1 led to a dramatic inhibition of 5'-strand resection (Figure 4A). In contrast to CtIP depletion, double depletion of WRN and EXO1 could not be rescued by excess MRN. Even after 3 h of incubation, DNA was still stable and no faster migrating products were detected. This was not due to the non-specific inactivation of the double depleted extract, because the resection defect could be efficiently complemented by the addition of recombinant EXO1 (Figure 4A).

The substrate used in the above experiment carried a 3'-label; limited resection of the 5'-strand might not have affected the mobility of the DNA and thus escaped detection. To further determine if excess MRN led to any resection of the 5'-strand, we determined the fate of the 5'-labeled DNA. As reported previously (8), double depletion of WRN and EXO1 also caused a significant inhibition of the removal of the 5'-label (Figure 4B). This inhibition was not significantly reversed by the addition of excess MRN. The kinetics of 5'-label removal was still much slower than that in the mock-depleted cytosol and there remained some 5'-label (~13%) remaining on the DNA after 3 h of incubation. Together, these data suggest that the mechanistic roles of WRN and EXO1 in 5'-strand resection are fundamentally distinct from those of MRN and CtIP and cannot be substituted by excess MRN.

MRE11 and CtIP can be bypassed by a DNA with a 3'-ss-tail

Genetic analyses in yeast are consistent with a two-step model for 5'-strand resection: initiation and extension. The initiation step is dependent on MRX and SAE2 by an unknown mechanism to produce a DNA with a

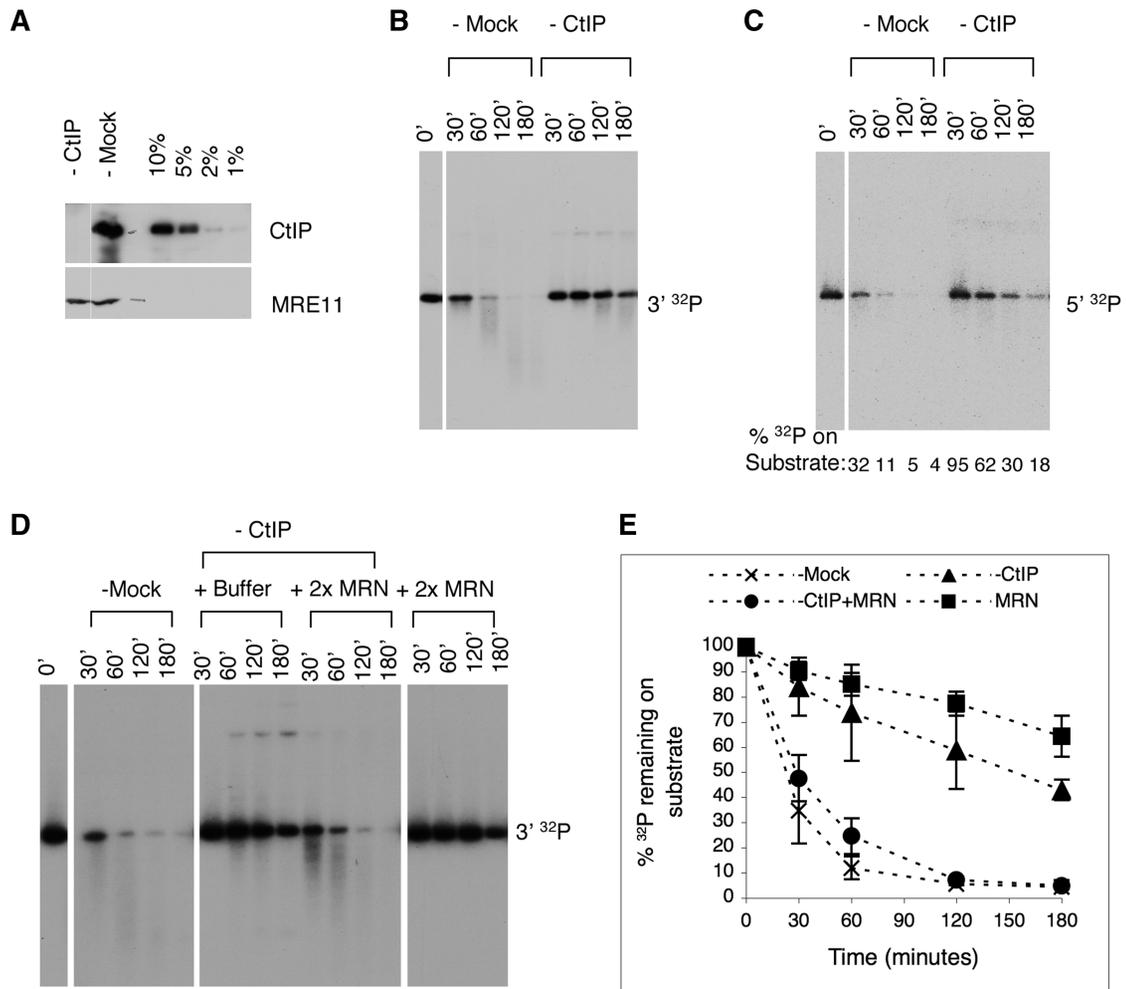


Figure 3. CtIP is important for 5'-strand resection but can be bypassed by excess MRN. (A) Efficiency of CtIP depletion. The standards for quantitation were cytosol loaded at different amounts relative to the depleted cytosol. (B) Effect of CtIP depletion on 5'-strand resection. 3'-³²P-labeled linear pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated in the CtIP-depleted or mock-depleted cytosol. Samples taken at the indicated times were analyzed by agarose gel electrophoresis. (C) Effect of CtIP depletion on the removal of the 5'-³²P label. Samples taken at the indicated times were analyzed by agarose gel electrophoresis. (D) Complementation of CtIP depletion with excess MRN. 3'-labeled substrate was incubated in the CtIP-depleted cytosol supplemented with MRN (2× the endogenous level = ca. 16 ng/μl) or buffer. Samples taken at the indicated times were analyzed by agarose gel electrophoresis. (E) Plot of the complementation assays. The amounts of 3'-³²P on the remaining substrates at the indicated times from three experiments were used to calculate the averages and standard deviations.

3'-ss-tail, which is then taken over by SGS1 and EXO1 for more extensive resection. To formally test this model, we analyzed the resection of a DNA that carried a 3'-ss-tail (ca. 200–300 nt), which is the putative product of the initiation step and the substrate of the extension step. This DNA was resected with a much faster kinetics than the blunt-ended DNA, as illustrated by the reaction with the mock-depleted cytosol in Figure 5A. The DNA was already converted into faster migrating products after 30 min and no longer detectable after 60 min, suggesting that the initiation step is rate limiting for resection. In MRE11-depleted or CtIP-depleted cytosol, the DNA was also efficiently resected. The kinetics of the MRE11 or CtIP-depleted cytosol was slower than that of the mock-depleted cytosol, but resection was still completed with high efficiency. These data suggest that MRE11 and CtIP are necessary for the initiation step but not the extension step of resection.

The bypass of CtIP by 3'-overhangs raised the possibility that this might be the explanation for the bypass of CtIP by excess MRN as shown in Figure 3. MRN displayed some weak activity to remove the 5'-label over time (Figure 4B), which might create 3'-overhangs. If this explanation is correct, one would expect that the kinetics of 5'-³²P removal by MRN should be faster than the kinetics of DNA resection in the CtIP-depleted extract supplemented with excess MRN. As shown in Figure 5B, the opposite is true. While practically all the DNA was already resected after 2 h of incubation in the CtIP-depleted cytosol supplemented with excess MRN, the purified MRN by itself could only remove a fraction of the 5'-label and at a much slower rate. There were still ca. 43 and 23% of the 5'-³²P remaining on the substrate after 2 and 3 h of incubation respectively. The bypass of CtIP by excess MRN is thus not simply by creating 3'-overhangs.

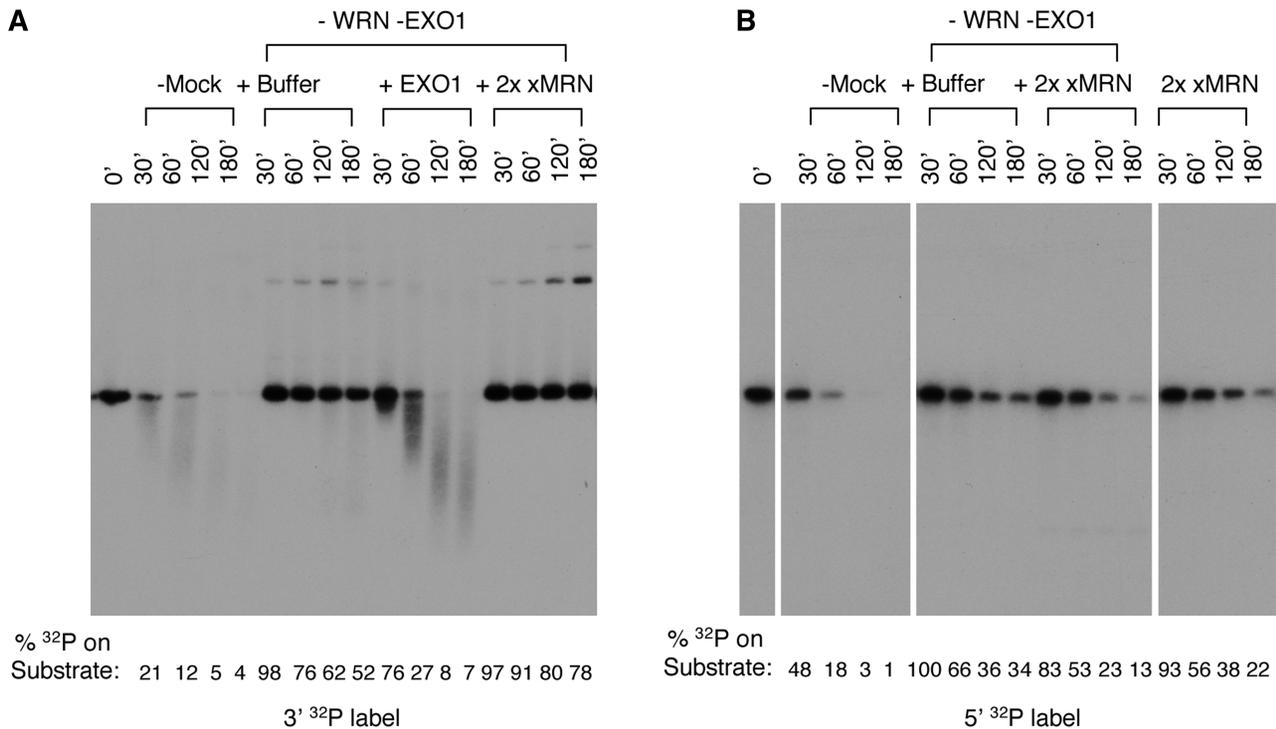


Figure 4. Excess MRN cannot bypass WRN and EXO1. **(A)** Effect of excess MRN on 5'-strand processing in WRN and EXO1 co-depleted cytosol. The 3'-³²P-labeled pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated in the mock-depleted cytosol or WRN and EXO1 co-depleted cytosol [supplemented with buffer, EXO1 or MRN (2×)]. Samples taken at the indicated times were treated with SDS/proteinase K and analyzed on a 1% TAE-agarose gel. **(B)** Effect of excess MRN on the removal of the 5'-³²P label in WRN and EXO1 co-depleted cytosol. 5'-labeled pBLP (1 ng/μl) was incubated in mock-depleted cytosol or WRN and EXO1 co-depleted cytosol (supplemented with buffer or 2× MRN). Samples were treated and analyzed similarly to that in A.

MRN is necessary and sufficient to reconstitute the WRN-DNA2-RPA pathway of 5'-strand resection with purified proteins

While our series of depletion studies have revealed that six proteins, MRN, CtIP, WRN, DNA2, RPA, and EXO1 play important roles in 5'-strand resection, an important mechanistic question was whether they were sufficient for resection. For technical reasons we were unable to express and purify active *Xenopus* CtIP protein, but the observation that it could be bypassed by excess MRN provided the rationale to reconstitute the two resection pathways with just MRN and WRN-DNA2-RPA or with MRN and EXO1. We have previously shown that purified WRN, DNA2 and RPA could resect DNA with a 3'-ss-tail in an ATP-dependent manner (7). However, they were very inefficient at resecting blunt-ended DNA (Figure 6A). Even after 2h of incubation, blunt-ended DNA remained stable. The addition of MRN dramatically stimulated the activity of WRN-DNA2-RPA towards resection of blunt-ended DNA. The DNA was degraded into faster migrating products that still retained the 3'-label, indicating that degradation proceeded in the 5'→3' direction. The amounts of proteins used were similar to those in the cytosol and the kinetics of resection was also very similar to that in the cytosol. These results thus showed that MRN was indeed sufficient to initiate the WRN-DNA2-RPA pathway under physiological conditions.

We next analyzed the contribution of individual components to resection. Reactions with various combinations of the four proteins and ATP were set up. As shown in Figure 6B, leaving out any component resulted in a strong inhibition of resection, suggesting that all four proteins as well as ATP were required for resection. However, in the absence of DNA2, a small amount of faster migrating DNA could be detected after long exposure. While this suggested that MRN could stimulate WRN's unwinding activity on blunt-ended DNA, the stimulation was very inefficient in the absence of DNA2.

We also examined if there were protein-protein interactions between MRN and the other proteins. MRN was co-incubated with each of the other three proteins and then immunoprecipitated with anti-MRE11 antibodies. As shown in Figure 6C, DNA2 and RPA were efficiently pulled down by the beads, suggesting that MRN could directly interact with these two proteins. Similar pull-down experiments failed to detect a re-producible interaction between MRN and WRN (data not shown). This failure might be due to steric hindrance from the antibody molecules. Indeed, it has been reported that human WRN interacts with human MRN (25). Furthermore, WRN is known to interact with RPA (7,26). It can thus still be part of the protein interaction network through which MRN assembles the 5'-strand resection machinery. Together, these data demonstrate that

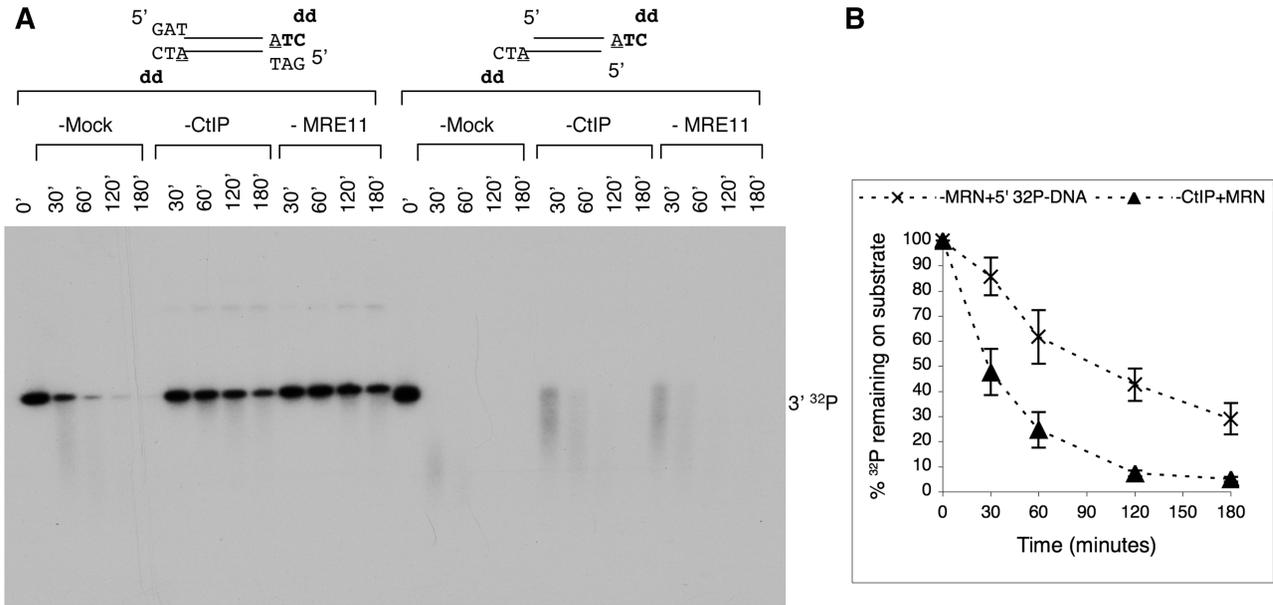


Figure 5. MRE11 and CtIP can be bypassed by a 3'-ss-tail. (A) 3'-labeled DNA (at the underlined nucleotide), either blunt ended or with a 3'-ss-tail was incubated with the indicated depleted cytosol. Samples taken at the indicated times were analyzed by TAE-agarose and the products were detected by exposure to an X-ray film. (B) Plot of the kinetics of 5'-³²P removal by the purified MRN (2×) and the kinetics of 3'-labeled DNA degradation in the CtIP-depleted cytosol supplemented with MRN (2×). Three sets of data were used to calculate the averages and standard deviations.

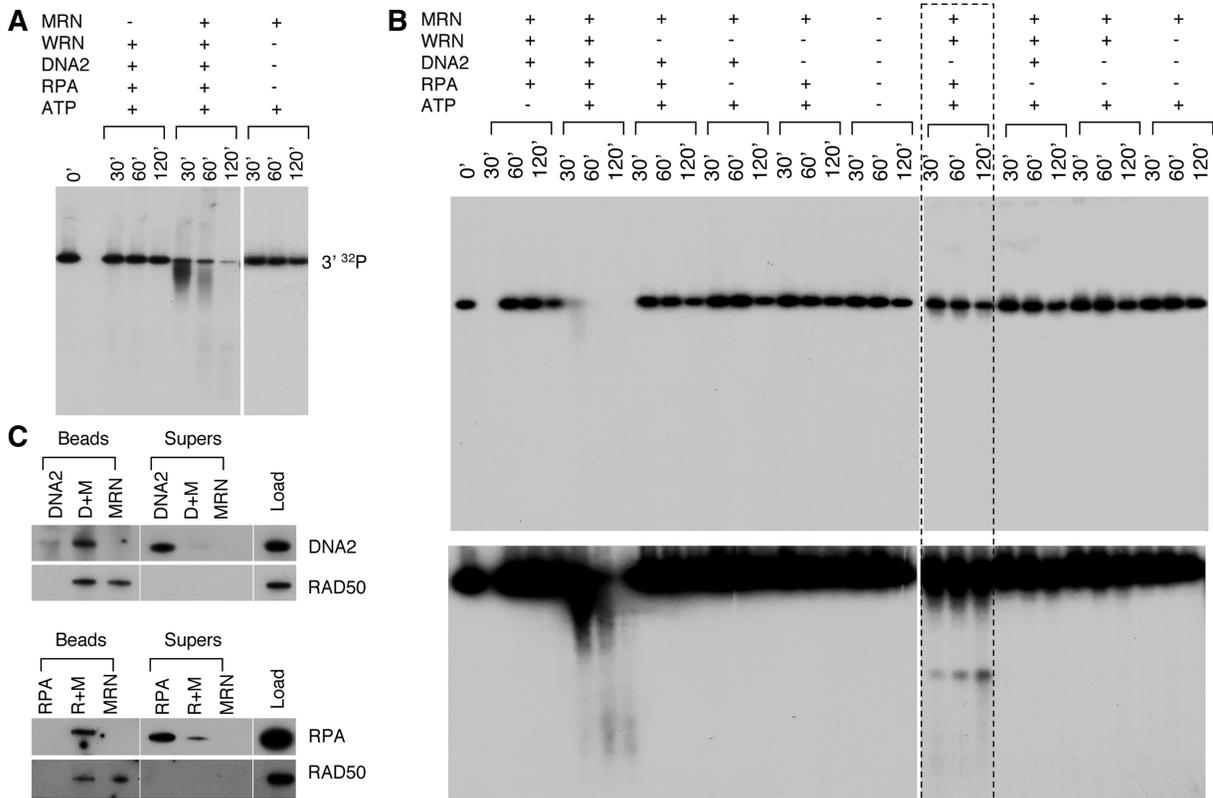


Figure 6. Purified MRN, WRN, DNA2 and RPA are sufficient to reconstitute 5'-strand resection. (A) MRN stimulated the activity of WRN-DNA2-RPA towards blunt-ended DNA. WRN, DNA2 and RPA were incubated with 3'-labeled DNA in the presence or absence of MRN. Samples taken at the indicated times were separated on a 1% TAE-agarose and the products were detected by exposure to an X-ray film. (B) The effect of each component on the reconstituted MRN-WRN-DNA2-RPA pathway. Various combinations of proteins and ATP were incubated with the 3'-labeled blunt-ended DNA. Samples taken at the indicated times were analyzed similarly to those in A. A long exposure was also presented to show the faint products. (C) Interactions of MRN with DNA2 and RPA. MRN was incubated with DNA2 or RPA and then immunoprecipitated by anti-MRN antibodies. The beads and supernatants were analyzed by Western blot with antibodies against DNA2, RPA, and RAD50.

MRN can interact with and stimulate the activity of the WRN-DNA2-RPA pathway for 5'-strand resection.

MRN interacts with xEXO1 and stimulates its 5'→3'-ds-DNA exonuclease activity

We next analyzed if MRN promotes the EXO1 pathway by stimulating its intrinsic 5'→3'-ds-DNA exonuclease activity. As shown in Figure 7A, in the presence of MRN, EXO1 rapidly degraded a 3'-labeled blunt ended DNA. Fast migrating intermediates still retained the label, which was consistent with the 5'→3' directionality of degradation. Interestingly, EXO1 was more active towards DNA with a 3'-ss-tail than blunt ended DNA (Figure 7B). It could degrade the former even in the absence of MRN, but this activity was still stimulated by MRN. This is consistent with the observation in Figure 5 showing that the kinetics of 3'-overhang DNA was slowed down in the absence of MRN.

One likely mechanism for MRN to stimulate EXO1 is by recruiting it to DNA. We first attempted a co-immunoprecipitation experiment but detected no significant interaction by this method. This might be the result of steric interference of the antibodies with protein-protein interaction. We thus used DNA magnetic beads to determine if MRN helps recruit EXO1 to DNA. EXO1 and MRN were incubated alone or together with DNA bound via one end to magnetic beads in the presence of EDTA to prevent degradation. When beads were pulled out and analyzed by Western blot, significantly more EXO1 was retained in the presence than in the absence of MRN (Figure 7C). Together these data suggest that MRN helps to recruit EXO1 to ds-DNA ends for efficient degradation of the 5'-strand.

DISCUSSION

In this study we combined the *Xenopus* egg extract system and purified proteins to analyze the function of MRE11 (as part of the MRN complex) in 5'-strand resection. Our major findings are: (i) MRE11 is important for 5'-strand resection of 'clean' DNA ends; (ii) in the absence of MRE11, even the first nucleotide at the 5'-end cannot be efficiently resected; (iii) CtIP is also important for 5'-strand resection but can be bypassed by excess MRN; (iv) excess MRN cannot bypass WRN and EXO1; (v) both MRE11 and CtIP can be bypassed if DNA carries a 3'-overhang; (vi) MRN interacts with and stimulates the WRN-DNA2-RPA pathway and all four proteins are required for efficient resection; and (vii) WRN helps recruit EXO1 to DNA and stimulates its 5'→3'-ds-DNA exonuclease activity. In total, six proteins: MRN, CtIP, WRN, DNA2, RPA and EXO1, have been identified to be important players in 5'-strand resection in *Xenopus* extract systems, demonstrating that this step of DSB repair is indeed highly conserved in eukaryotes. These findings greatly increase our understanding of 5'-strand resection and reconcile the many conflicting observations on MRE11's function in this important process.

The importance of MRN to 5'-strand resection has been an enigma. Early studies in asynchronous cultures of yeast cells showed only a 2-fold decrease in resection in the absence of MRE11 function (9). Later studies suggested that MRX affects only some ends, while other ends are still resected by SGS1-DNA2 or EXO1 at the normal rate. It is unclear why there should be this dichotomy of dependence on MRX. Our study demonstrated that MRN is indeed essential for 5'-strand resection of blunt or nearly blunt-ended DNA. However, if an end carries a

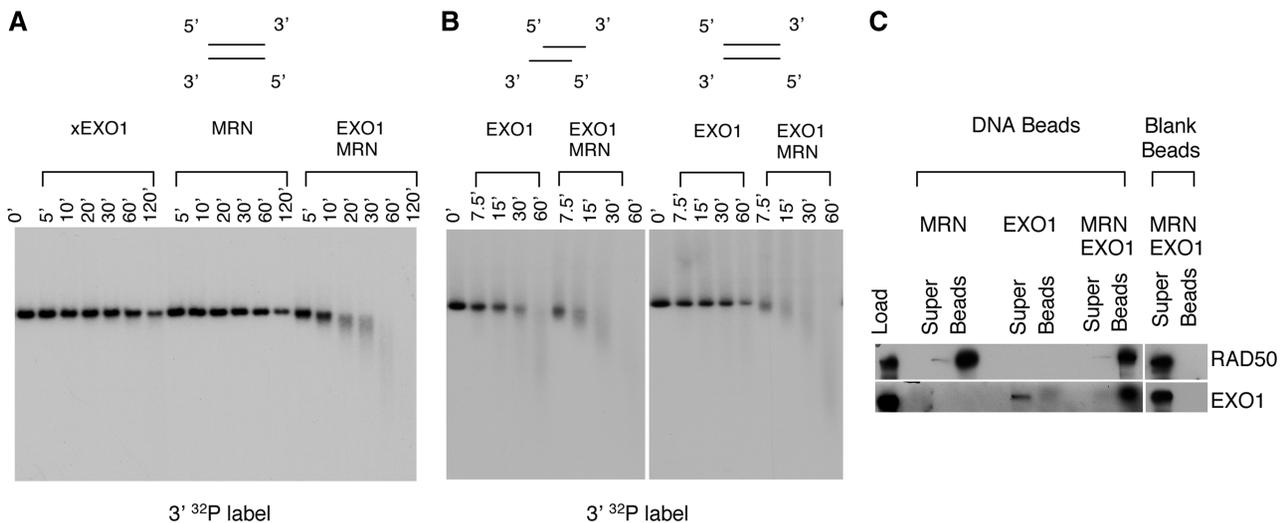


Figure 7. MRN stimulates the activity of EXO1. (A) EXO1 and MRN, alone or together, were incubated with 3'-³²P labeled blunt-ended DNA. Samples taken at the indicated times were separated on a 1% TAE-agarose and the products were detected by exposure to an X-ray film. (B) EXO1 and MRN, alone or together, were incubated with 3'-labeled DNA with a 3'-overhang (ca. 200–300 nt). Samples taken at the indicated times were separated on a 1% TAE-agarose and the products were detected by exposure to an X-ray film. (C) MRN helps recruit EXO1 to DNA ends. EXO1 and MRN, alone or together, were incubated with 1.5 kb DNA anchored via biotin at one end onto Streptavidin magnetic beads. A reaction with EXO1, MRN and blank beads was also set up as a control. The beads and supernatant fractions were analyzed for EXO1 and RAD50 by Western blot analysis.

3'-overhang, MRN is no longer essential. Ends with 3'-overhangs are expected to arise during S phase as the result of replication forks colliding with strand breaks, including the DSBs generated by restriction enzymes. The nature of leading and lagging strand synthesis predicts that the DSB upstream of the break is likely to be blunt ended, but the DSB downstream of the break should carry a 3'-overhang. The dichotomy of dependence on MRX might be the result of different structures of ends. Consistent with this interpretation, MRX has been reported to be essential for resection during G2 phase (27,28). There is no DNA replication during G2 phase, so DSBs generated by restriction enzymes are expected to remain nearly blunt ended and thus, as demonstrated by our data, require MRX for resection.

Genetic studies suggested that MRN is functionally intimately related to CtIP/SAE2, but the nature of this relationship has been unclear. In fact, the role of CtIP/SAE2 in end resection is itself also an enigma. While CtIP/SAE2 is clearly important in yeast, human cells, and *Xenopus* egg extracts, it is not required in several reconstitution experiments with purified proteins (13,14,24). Our study provides a rational explanation for this discrepancy: CtIP can be bypassed by excess MRN. Even a 2-fold increase in the level of MRN can do so. In contrast to CtIP, the function of WRN and EXO1 cannot be bypassed by excess MRN. This suggests that there is a fundamental difference in the mechanistic roles of WRN/EXO1 and CtIP. As a helicase and a nuclease, WRN and EXO1 are the mechanical components that are physically responsible for the resection of ends. It is reasonable that their function cannot be replaced by MRN. CtIP acts more like a regulatory factor for MRN, and is dispensable if more MRN is present. CtIP can interact with MRN and stimulate its ss-DNA endonuclease activity, although it is still unclear how this activity contributes to 5'-strand resection (16). CtIP is also found in a complex that contains BRCA1, which has also been implicated in resection and has E3 ubiquitin ligase activity and might modify MRN to facilitate resection (17). Future studies are required to elucidate the nature of regulation of MRN by CtIP.

Of the two major pathways for 5'-strand resection, the EXO1 pathway is simple and conserved in yeast, *Xenopus* and human cells. The pathway catalyzed by the RecQ helicases, however, is complicated by the fact that there are multiple RecQ family members in higher eukaryotes, all of which play critical roles in genome maintenance yet each displays a distinct phenotype when inactivated (29). It is unclear what determines which RecQ helicase is used for 5'-strand resection. Indeed, in human cells, BLM has been reported to be the major RecQ-type helicase in generating RPA foci (presumably as the result of 5'-strand resection) at DSBs (3). Furthermore, in the reconstitution system with purified human proteins, it was reported that only BLM could catalyze resection (24). However, the *in vivo* experiments did not have the resolution to rule out a role for WRN and the reconstitution experiment testing WRN was done in the absence of MRN. Our studies clearly showed that WRN depletion caused a strong inhibition of resection. In the presence

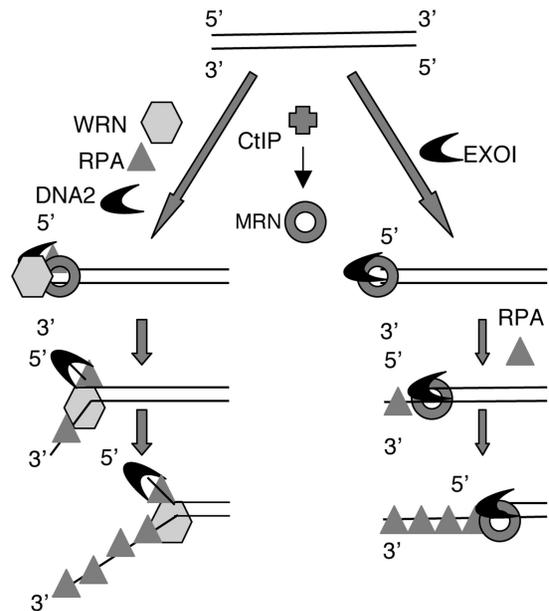


Figure 8. Model for the two pathways of 5'-strand resection. MRN is directly involved in the initiation of resection while CtIP stimulates the activity of MRN. The other resection proteins are involved in both the initiation step and the extension step.

of MRN, but not in its absence, WRN, DNA2, and RPA are very robust in executing 5'-strand resection. Together our studies strongly argue that the failure to detect WRN's activity in the previous reconstitution experiment was due to the omission of MRN.

Based on the observations made in this study and our previous studies and studies in other model systems, we propose the following model for 5'-strand resection (Figure 8). MRN is directly required for the initiation of both the WRN-DNA2-RPA pathway and the EXO1 pathway. CtIP acts indirectly by increasing the activity of MRN. For the WRN-DNA2-RPA pathway, MRN binds to DNA ends and interacts with DNA2 and RPA, and RPA also interacts with WRN and DNA2. The network of interactions among all four proteins helps the building of a resection machinery at DNA ends. Ends are unwound by WRN and the 5'-ss-tails are then degraded by DNA2. Both reactions are stimulated by RPA. MRN's role is limited to the initiation step. For the EXO1 pathway, MRN also helps to recruit it to DNA ends and stimulates its intrinsic ds-DNA 5'→3' exonuclease activity. In addition, MRN plays a non-essential role in facilitating the processivity of EXO1. Further studies will be needed to test this model and elucidate the functional roles of the various enzymatic activities endowed by MRN, in particular its nuclease activity.

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