

Mechanistic analysis of *Xenopus* EXO1's function in 5'-strand resection at DNA double-strand breaks

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ABSTRACT

The processing of DNA double-strand breaks (DSBs) into 3' single-stranded tails is the first step of homology-dependent DSB repair. A key player in this process is the highly conserved eukaryotic exonuclease 1 (EXO1), yet its precise mechanism of action has not been rigorously determined. To address this issue, we reconstituted 5'-strand resection in cytosol derived from unfertilized interphase eggs of the frog *Xenopus laevis*. *Xenopus* EXO1 (xEXO1) was found to display strong 5'→3' dsDNA exonuclease activity but no significant ssDNA exonuclease activity. Depletion of xEXO1 caused significant inhibition of 5' strand resection. Co-depletion of xEXO1 and *Xenopus* DNA2 (xDNA2) showed that these two nucleases act in parallel pathways and by distinct mechanisms. While xDNA2 acts on ssDNA unwound mainly by the *Xenopus* Werner syndrome protein (xWRN), xEXO1 acts directly on dsDNA. Furthermore, xEXO1 and xWRN are required for both the initiation stage and the extension stage of resection. These results reveal important novel information on the mechanism of 5'-strand resection in eukaryotes.

INTRODUCTION

Among the numerous types of damages to the genome, DNA double-strand breaks (DSBs) are the most deleterious to cells. Unrepaired or improperly repaired DSBs would cause chromosomal deletions, translocations or duplications, leading to cell death or oncogenic transformation (1). In eukaryotes, there are three major DSB repair pathways: non-homologous end joining (NHEJ), homologous recombination (HR) and single-strand annealing (SSA) (2). NHEJ is accomplished by the direct joining of ends, usually after some minor polishing of ends to remove damaged nucleotides. HR repairs DSBs by invading a homologous sequence and copying the missing information. If a break occurs between two direct repeats,

it can also be repaired by SSA and the final product retains effectively only one of the two repeats. HR and SSA are both dependent on sequence homology and thus also collectively referred to as homology-dependent DSB repair (HDR).

The crucial event that dictates the choice of DSB repair pathway is the initial processing of DNA ends. While NHEJ is usually associated with minimal processing, HR and SSA require more extensive degradation of 5'-strands to generate 3' ss-tails. 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 mechanisms responsible for 5'-strand resection in various eukaryotic systems are beginning to be elucidated. Using *Xenopus* nucleoplasmic extracts (NPE) as the model system, we have discovered that one major pathway consists of two coupled reactions: an ATP-dependent unwinding of ends and the subsequent ATP-independent degradation of 5' ss-tails, resulting in 3' ss-tails as the final product (3). The major DNA helicase for end unwinding is the *Xenopus* Werner syndrome protein (xWRN), a RecQ type DNA helicase, and the major 5'→3' ssDNA exonuclease is the *Xenopus* DNA2 protein (xDNA2) (3,4). Both steps are dramatically stimulated by replication protein A (RPA), which is also required for efficient 5'-strand processing (5). This coupled mechanism of a RecQ-type DNA helicase and a 5'→3' ssDNA exonuclease appears to be highly conserved in other organisms. In *Escherichia coli*, the prototype RecQ helicase can act with RecJ, a 5'→3' ssDNA exonuclease, to degrade 5'-strands (6). In budding yeast *Saccharomyces cerevisiae*, the RecQ-type helicase SGS1 participates in the 5'-strand resection at DSBs (7–9) and DNA2 acts in the same epistatic pathway as SGS1 (9). In human U2OS cells, the Bloom syndrome protein (BLM), one of the five mammalian RecQ-type helicases, appears to be the dominant helicase to promote end processing of camptothecin induced DSBs (7).

In addition to the RecQ-DNA2 pathway, studies in yeast and human cells have suggested that another nuclease, EXO1, a member of the RAD2 nuclease family, also participates in 5'-strand resection (7–9).

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Double mutants of EXO1 and SGS1 or DNA2 in yeast and simultaneous knockdown of EXO1 and BLM by siRNAs in human cells cause much more dramatic inhibition of ss-DNA generation than single mutants or knockdowns. While the evidence for *Xenopus* EXO1's (xEXO1) involvement in 5'-strand resection is compelling, its mechanism of action remains to be rigorously defined. In theory, EXO1 might act directly on dsDNA to degrade the 5'-strand or, similarly to DNA2, act in conjunction with a DNA helicase to degrade the 5'-ssDNA after the ends are unwound. Yeast EXO1 (yEXO1) has been reported to possess both 5'→3' dsDNA and 5'→3' ssDNA exonuclease activities (10,11). Human EXO1 (hEXO1) has been reported to possess 5'→3' dsDNA exonuclease, 5'→3' ssDNA exonuclease, and 5' flap endonuclease activities (11,12). The ssDNA exonuclease activity for both yEXO1 and hEXO1 is less than the ds-exonuclease activity (10,11). Yet ssDNA exonucleases (i.e. DNA2) are often stimulated by ssDNA binding proteins like RPA, which was not included in those studies. Interestingly, the 5'→3' ds-exonuclease activity of hEXO1 is stimulated by BLM (13), but this stimulation is independent of ATP and DNA end unwinding. In addition, both the 5'→3' ds-exonuclease activity and the 5' flap endonuclease activity of human EXO1 have been reported to be stimulated by WRN (14). However, if ATP is present, a flap-like substrate is either cleaved by EXO1 or unwound by WRN, suggesting that EXO1 cannot access the ss/dsDNA junction if WRN already occupies it to unwind DNA. It is difficult to deduce based on enzymatic characterizations alone how EXO1 accomplishes 5'-strand processing.

Another puzzling aspect of EXO1 action in 5'-strand resection is that, while the purified EXO1 by itself possesses nuclease activity, studies in *S. cerevisiae* suggest that the EXO1 and SGS1 pathways appear to act downstream of MRE11-RAD50-XRS(MRX) and SAE2 (8,9). The double mutant of *exo1 sgs1* still showed residual 5' resection of a few hundred nucleotides by a MRX- and SAE2-dependent reaction. It is unclear how the enzymatic activities of MRX and SAE1 themselves can accomplish this initial resection. MRE11 shows ssDNA endonuclease activity and 3'→5' dsDNA exonuclease activity, and SAE2 displays DNA hairpin endonuclease activity (15–17). These activities are incompatible with the 5'→3' directionality of strand resection at DSBs. The interpretation of the genetic data is potentially complicated by the leakiness of *exo1 sgs1* double mutants and/or the acquisition of compensating mutations in the mutant strains. As such, the actual degradation of the initial region of DNA might still be carried out by EXO1 and SGS1-DNA2, but in an MRX and SAE2-dependent way. Notably, homologues of MRX (MRN) and SAE2 (CtIP) are also important for strand resection in mammalian cells (18–20) and *Xenopus* egg extracts (21,22). The mechanistic relationship among EXO1, WRN-DNA2, MRN and CtIP is thus most likely conserved in the *Xenopus* system, making it a powerful tool to determine if EXO1 and WRN-DNA2 are really dispensable for the initiation processing of DNA ends.

In this study, we took advantage of the *Xenopus* egg extract system to define rigorously the mechanistic role of EXO1 in 5'-strand resection. We first established membrane-free cytosol derived from unfertilized interphase *Xenopus* eggs as a more convenient system than NPE for studying 5'-strand resection. We found that purified xEXO1 displayed strong 5'→3' exonuclease activity against dsDNA but not against ssDNA, even in the presence of RPA. Consistent with the enzymatic properties of xEXO1, immunodepletion of EXO1 caused a significant inhibition of 5' resection of ds-DNA but showed no effect on ssDNA degradation. When xEXO1 was co-depleted with xWRN or xDNA2, 5'-strand resection was inhibited much more strongly than single depletions of these proteins. By using a DNA with a ³²P label placed at the 5'-end, we found that the undegraded DNA in the doubly depleted cytosol still retained the ³²P label, indicating that xEXO1 and xWRN-xDNA2 participate in both the initiation stage and the extension stage of resection. Together, these results show that the two pathways for 5'-strand resection are conserved in the *Xenopus* system and reveal important insights into their distinct mechanism of action in DSB repair.

MATERIALS AND METHODS

Extract preparation

Membrane-free cytosol was prepared from unfertilized *Xenopus* eggs following the standard protocol (23).

Expression and purification of recombinant xEXO1

The DNA containing the full-length xEXO1 open reading frame (ORF) was purchased from Open Biosystems (ThermoFisher, AL, USA). After confirmation by sequencing, the xEXO1 ORF was subcloned into a modified pFastBac (Invitrogen, CA, USA) for expression in SF9 cells. The nuclease-dead mutant xEXO1 (D173A) was prepared using the QuikChange site-directed mutagenesis kit of Stratagene (Agilent, CA, USA). The two oligonucleotides used for mutagenesis are: 5'-GCTATA ATTACAGAAGATTCTGCTCTTTTAGCATTGGTT GC-3' and 5'-GCAACCAAATGCTAAAAGAGCAGA ATCTTCTGTAATTATAGC-3'. SF9 cells expressing the two recombinant proteins were collected and nuclear extracts were prepared following the standard protocol (Invitrogen). The extracted nuclear proteins were first separated by HiTrap Q Sepharose (GE, NJ, USA). Fractions eluted between 100–150 mM NaCl contained significant amounts of xEXO1 but minimal activity of contaminating endogenous nucleases were then loaded on HiTrap Heparin Sepharose (GE, NJ). Peak fractions of xEXO1 (430–460 mM NaCl) were concentrated with Amicon Ultra-4 (MWCO = 10k; Millipore, MA, USA) and saved as small aliquots at –80°C.

Antibody production and purification

The full-length xEXO1 ORF was subcloned into pET28 and pGEX expression vectors to be expressed as His-xEXO1 and GST-xEXO1 fusion proteins. The two

fusion proteins were expressed in bacterial BL21(DE3) cells and the resulting inclusion bodies were purified. The His-xEXO1 fusion protein was further separated on a SDS-PAGE gel and the band corresponding to the full-length His-xEXO1 was isolated. Gel slices containing ~1 mg of antigen were mixed with Incomplete Freund's Adjuvant and injected into two New Zealand White rabbits (Covance, NJ, USA). The procedure was repeated at 21-day intervals for a total of six injections. Purification of specific anti-xEXO1 antibodies was achieved with an affinity column constructed with the GST-xEXO1 fusion protein following the published protocol (24). Other antibodies used in the study, rabbit anti-xDNA2 and rabbit anti-xWRN, were produced and purified following the same procedure (4,25).

Immunodepletion

To deplete xEXO1, cytosol {40 μ l + 20 μ l ELB [10 mM HEPES (pH7.5), 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 1.5 μ g of the affinity-purified rabbit anti-xEXO1 antibodies or buffer 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 xDNA2 and xWRN, singly, doubly or in combination with xEXO1, followed the same procedure with the corresponding antibodies.

Nuclease assays

The DNA substrates for the nuclease assays were prepared as previously described (3). ³²P-labeled and biotinylated 48-mer oligonucleotides, in either the ss-form or the ds-form, were coated onto Streptavidin paramagnetic beads following the manufacturer's instruction (Invitrogen). The substrates were designated as 5' or 3' depending on the open end on the ³²P-labeled strand. A typical nuclease reaction contained 5 μ l protein to be assayed (in A250 buffer or equivalent buffer), 5 μ l ELB buffer, and 0.1 μ l of oligonucleotide beads (1 ng/ μ l for dsDNA beads and 0.5 ng DNA/ μ l for ssDNA beads). After incubation at room temperature (22–25°C) for 60 min with rotation, the reactions were stopped with equal volume of 2% SDS–25 mM EDTA, heated at 95°C for 15 min, and analyzed by 8% TAE-PAGE.

DNA 5'-strand resection assays

The DNA substrates for studying DNA strand resection was prepared by digesting the plasmid pBLP with HindIII or BamHI and filling the ends with dGTP, dTTP, ddCTP and ³²P dATP. ssDNA was prepared by heat denaturing a 3'-labeled pBS plasmid (~3 kb in size) and then immediately chilling it on ice. A typical DNA end processing 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), 0.1 μ l DNA (75 ng/ μ l), 0.15 μ l 10 mM ddCTP, 1.75 μ l appropriate protein (for complementation) or ELB buffer (total volume = 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, the samples were brought up to 10 μ l with H₂O and supplemented with 1 μ l proteinase K (10 mg/ml). After incubation at room temperature for at least 2 h, the samples were analyzed by 1% TAE-agarose gel electrophoresis. Gels were stained with SYBR Gold (Invitrogen) for detection of total DNA and then dried for exposure to Phosphorimager (Fuji) or film to detect ³²P.

RESULTS

Establishment of cytosol as a convenient system for studying 5'-strand resection

We have previously used NPE, which is extracted from nuclei reconstituted in crude interphase *Xenopus* egg extracts, as the model system to study SSA and 5'-strand resection. While extremely potent, NPE is unfortunately difficult to prepare and the quantity obtained is limited. In addition, the potency of NPE owes to the high concentration of replication and repair proteins, but this also makes it difficult to deplete a protein of interest. We thus explored the possibility of using the cytosol of interphase *Xenopus* eggs, which is easy to prepare (by a simple ultracentrifugation of crude extracts) and deplete (repair proteins being 5–10 \times less concentrated than in NPE), to reconstitute 5'-strand resection. Previous studies have shown that cytosol contains robust activity for Ku-dependent NHEJ (26,27). When a linear DNA with incompatible ends (to prevent simple religations) was incubated in cytosol, it was rapidly and efficiently repaired into supercoiled monomers, relaxed monomers, dimers and larger multimers (Figure 1A). NHEJ could be inhibited if a dideoxynucleotide (dideoxycytidine or ddC) was placed at the end of the linear DNA or added to the extract (Figure 1A). Interestingly, in this situation, the fate of the unrepaired DNA in cytosol depended on the concentration of DNA (Figure 1B). At 5 ng/ μ l (~1.6 \times 10⁹ ends/ μ l for a 5.7 kb DNA), the unrepaired DNA was stable, with only a small fraction degraded during the three hours of incubation at room temperature (detected as the faster migrating smear by Southern blot hybridization). In contrast, at 1 ng/ μ l (~3 \times 10⁸ ends/ μ l), the unrepaired DNA was almost completely degraded within 3 h of incubation. Processing intermediates could also be detected by a ³²P-label placed immediately inside the 3'-terminal ddC. As shown in Figure 1C, despite the proximity of the ³²P-label to the 3'-end, the end processing intermediates still retained the label. In contrast, for DNA with ³²P placed at the 5'-end, no intermediates were detectable, indicating that the label was removed as soon as DNA processing started (Figure 1C). These properties showed that linear DNA degradation in cytosol, as in NPE, proceeded in the 5'→3' direction. Further characterizations revealed that the degradation was dependent on ATP (data not shown). Because of the convenience of preparing and depleting cytosol, we thus switched to this system to study 5'-strand resection. (1 ng/ μ l was used as the standard concentration for end processing experiments with cytosol. In addition, Southern blot hybridization and 3'-³²P label were used interchangeably to detect DNA end processing).

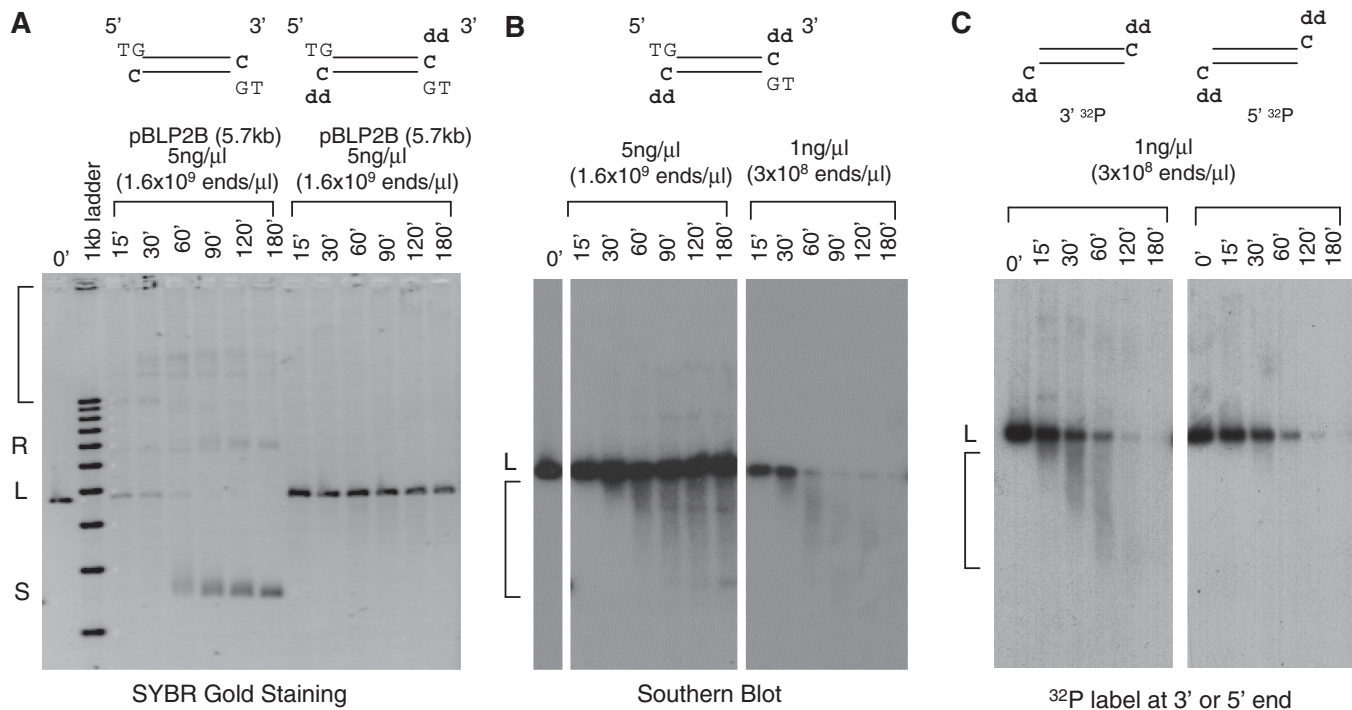


Figure 1. Establishment of cytosol as a system for studying 5'-strand processing. (A) A dideoxynucleotide at the 3'-end blocks NHEJ. A 5.7kb linear DNA (pBLP) with either dC or ddC at the 3'-end was incubated in cytosol at 5ng/μl. Samples were taken at the indicated times, treated with SDS-proteinase K and separated on a 1% TAE-agarose gel. DNA was detected by SYBR Gold staining. L: linear substrate. S: supercoiled monomer product. Bracket: dimers, trimers and multimers. (B) Effect of DNA concentration on 5'-strand processing. DNA with ddC-terminated 3'-ends was incubated in cytosol at 5ng/μl and 1ng/μl concentrations. Samples were processed as in (A), transferred to a Nylon membrane, and detected by Southern hybridization with a ³²P-labeled probe. L: linear substrate. Bracket: intermediates. (C) Differential fate of 5'- and 3'-³²P-label during 5' strand processing. 5'- or 3'-labeled DNA with ddC-terminated 3'-ends was incubated in cytosol for the indicated times and separated by gel electrophoresis as in (A). The gel was dried and the ³²P signal was detected by exposure to an X-ray film.

Xenopus EXO1 (xEXO1) possesses 5'→3' exonuclease activity toward dsDNA but not ssDNA

Studies in yeast *S. cerevisiae* and human cells have shown that EXO1 constitutes one pathway for 5'-strand resection at DSBs. To determine if this pathway is conserved in the *Xenopus* system and, if so, investigate the mechanistic role of xEXO1 in it, we identified the gene encoding xEXO1 in the data base (gi:54035217). Sequence analysis showed that xEXO is 734 amino acids in length and displays strong homology to human EXO1 (51% identity and 62% and 64% similarity to hEXO1a and hEXO1b, respectively). Recombinant xEXO1 and a mutant that changed the catalytic amino acid (D173A) were expressed in insect cells and purified (Figure 2A). They were assayed for nuclease activities toward four different substrates: 5'-dsDNA, 5'-ssDNA, 3'-dsDNA and 3'-ssDNA. These were ³²P-labeled 48-mer oligonucleotides anchored on Streptavidin paramagnetic beads, leaving only one end of the labeled strand accessible to nucleases. (Directionality was thus assigned based on the accessible end of the labeled strand). As shown in Figure 2B, wild-type xEXO1, but not mutant xEXO1 displayed strong activity towards the 5' dsDNA. After 1 h of incubation at room temperature, the substrate was almost completely degraded. 5' ssDNA, in contrast, was not degraded by xEXO1, even in the presence of the ssDNA binding

protein RPA (Figure 2C). With 3' ds- and ssDNA, wild-type xEXO1 caused a small amount of degradation, but this weak activity was completely blocked by RPA (Figure 2D). Collectively, these enzymatic studies showed that xEXO1 possesses strong 5'→3' dsDNA exonuclease activity, an activity compatible with its potential role in 5'-strand resection at DSBs.

xEXO1 is important for 5'-strand processing

To determine if xEXO1 is indeed involved in 5'-strand resection, we prepared antibodies against recombinant xEXO1 expressed in bacteria. The anti-xEXO1 serum, but not the pre-immune, detected a protein of ~90 kd in cytosol (Figure 3A). This was slightly larger than the calculated molecular weight of 82.2 kd [most likely due to the high pI value (=9.1)] but just slightly smaller than the recombinant His-xEXO1 protein. Anti-xEXO1 antibodies were purified and then used to deplete xEXO1 from cytosol. As shown in Figure 3B, xEXO1 could be depleted to a level below detection (>98% depletion). The substrate for the DNA degradation assay was a 5.7kb linear pBLP DNA that had been digested with BamHI and filled in with dGTP, ³²P-dATP, dTTP and ddCTP. This DNA could not be efficiently repaired by NHEJ due to the ddC at the 3'-end. In mock-depleted cytosol, it was rapidly degraded, as detected by both

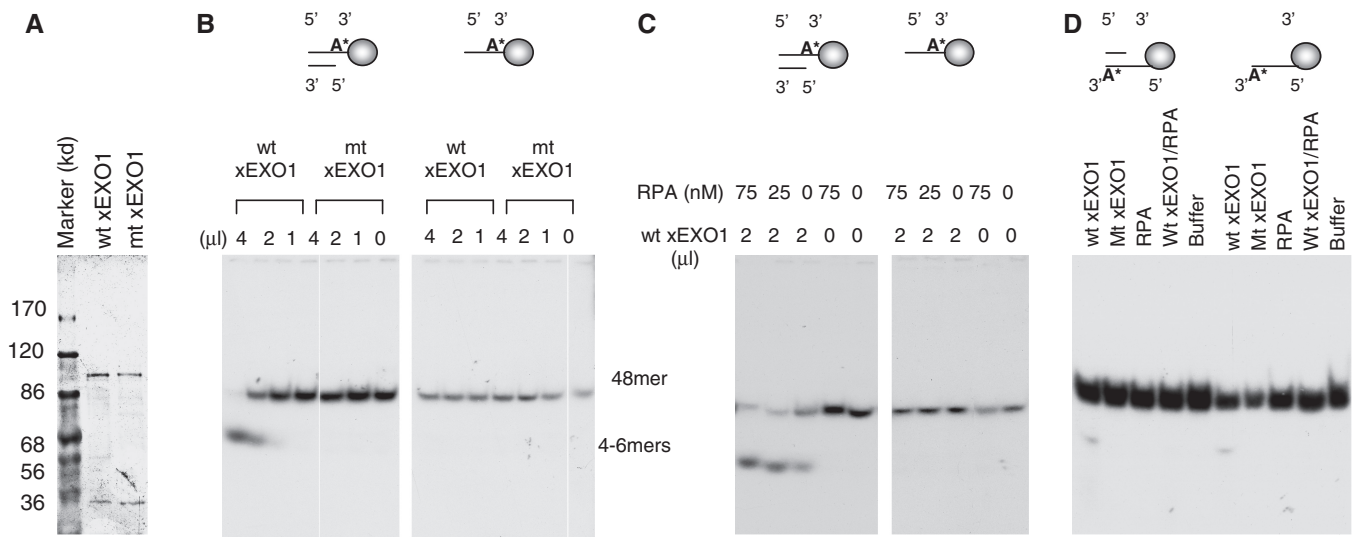


Figure 2. Characterization of xEXO1's nuclease activity. (A) A SDS-PAGE showing the purified recombinant wild-type and mutant xEXO1. (B) Nuclease assay of recombinant xEXO1 with different DNA substrates. The substrates were 48-mer oligonucleotides in either ss- or ds-form attached to magnetic beads, leaving the 5'-end (of the ³²P-labeled strand) accessible to the nuclease. After 1 h of incubation at room temperature, the reactions were terminated with 1% SDS, heated at 95°C for 15 min, and then separated on an 8% TAE-PAGE. (C) Effect of RPA on the 5'→3' exonuclease activity of xEXO1. Different amounts of RPA and xEXO1 were incubated with the ³²P-labeled 5' accessible ss- or ds-48-mer beads for one hour at room temperature. The analysis was similar to that in (A). (D) Determination of the 3'→5' exonuclease activity of xEXO1. Four microliters of xEXO1 was incubated with ³²P-labeled 3' accessible ss- or ds-48-mer beads in the presence or absence of RPA (75 nM) at room temperature for 1 h. The analysis was similar to that in (A).

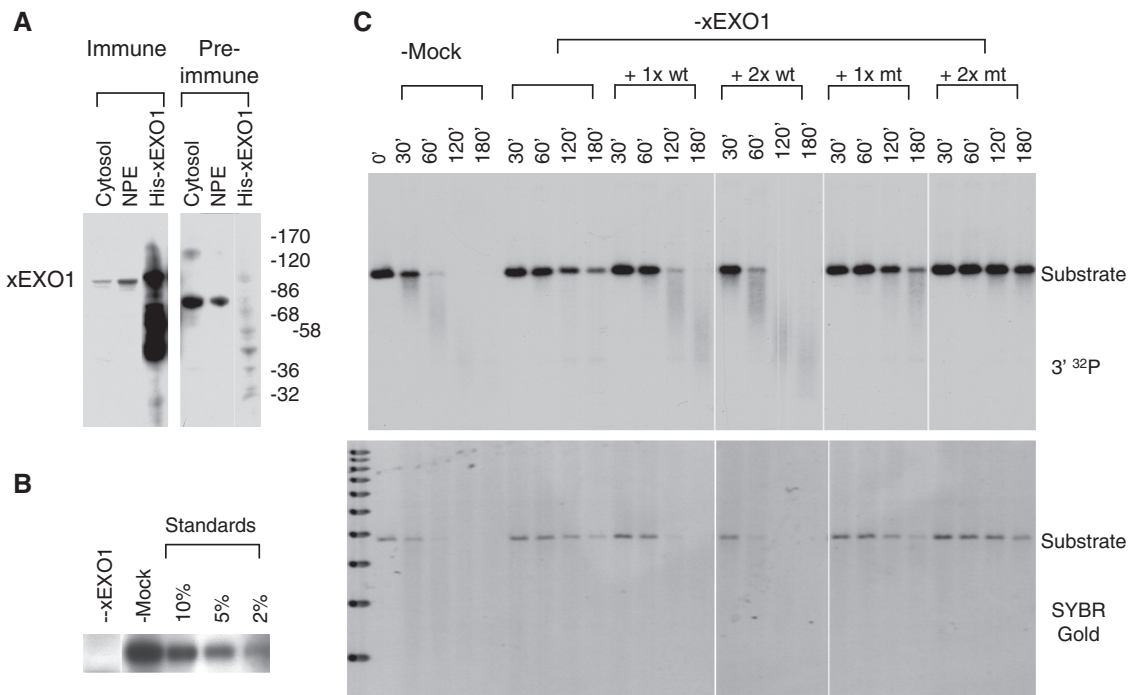


Figure 3. Effect of xEXO1 on 5'-strand processing. (A) Detection of xEXO1 in cytosol and NPE by western blot with anti-xEXO1 bleed (immune) and pre-bleed (pre-immune). (B) Efficiency of xEXO1 depletion. The quantitation standards for western blot were untreated cytosol at different amounts relative the depleted cytosol. (C) Effect of xEXO1 depletion on 5'-strand processing. 3'-³²P-labeled linear pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated in xEXO1-depleted or mock-depleted cytosol. Four additional reactions of xEXO1-depleted cytosol were supplemented two different levels of wild-type (wt) and mutant (mt) recombinant xEXO1. Samples taken at the indicated times were treated with SDS-proteinase K and separated on 1% TAE-agarose gel. The gel was stained with SYBR Gold and then dried for exposure to an X-ray film.

SYBR Gold DNA staining and ^{32}P label on the 3'-end (Figure 3C). In contrast, in xEXO1-depleted cytosol, DNA degradation was inhibited significantly (but not completely) (Figure 3C). Even after 180 min of incubation, there was still a significant amount of DNA undegraded. The xEXO1 depletion effect was specifically due to the removal of xEXO1 rather than cross-reacting proteins because it could be reversed by the addition of the wild-type recombinant xEXO1 protein (Figure 3C). Mutant xEXO1, in contrast, showed little (at 1 \times concentration) or even some inhibitory (dominant negative) effect (at 2 \times concentration). Together, these results demonstrated that xEXO1 is indeed an important player in 5'-strand resection in *Xenopus* cytosol.

xEXO1 and xDNA2 act in parallel pathways

The depletion of xEXO1 from cytosol did not completely block 5' resection, suggesting that there is another factor(s) that can also contribute to DNA degradation. We previously found that depletion of xDNA2 from NPE caused significant but incomplete inhibition of 5'-strand resection in the *Xenopus* NPE system (4). Similarly, using binding of RPA to DNA magnetic beads (presumably through the ss-DNA generated by strand resection) as the readout, Wawrousek *et al.* (22) found that depletion xDNA2 from *Xenopus* egg extracts also caused

a partial inhibition of RPA binding. We thus examined if xDNA2 is the nuclease that acts in parallel to xEXO1 in 5'-processing in cytosol. Cytosol was immunodepleted of xDNA2, either singly or in combination with xEXO1 (Figure 4A). As shown in Figure 4B, xDNA2 depletion from cytosol, like that from NPE indeed caused a significant but incomplete inhibition of 5'-strand resection. The inhibition was specific because it could be reversed by the addition of the purified xDNA2. Double depletion of xDNA2 and xEXO1 caused a much stronger inhibition of DNA degradation than single depletions of either xDNA2 or xEXO1 (Figure 4C). Even after 3 h of incubation, there was still on average 47.7% (SD = 2.5%) of the DNA substrate remaining in the cytosol depleted of xEXO1 and xDNA2 (Figure 4D). This was significantly more than those depleted of xEXO1 only (31%; SD = 2.6%; $P = 0.0076$) or xDNA2 only (20.7%; SD = 4.1; $P = 0.0051$). These results showed that xEXO1 and xDNA2 are both involved in 5'-strand resection and, similarly to their yeast homologues, act in parallel pathways.

xEXO1 does not act through ssDNA intermediates

A key question about EXO1 is the mechanism by which it executes 5'-strand resection. xDNA2, which has strong RPA-dependent 5' \rightarrow 3' ssDNA exonuclease activity,

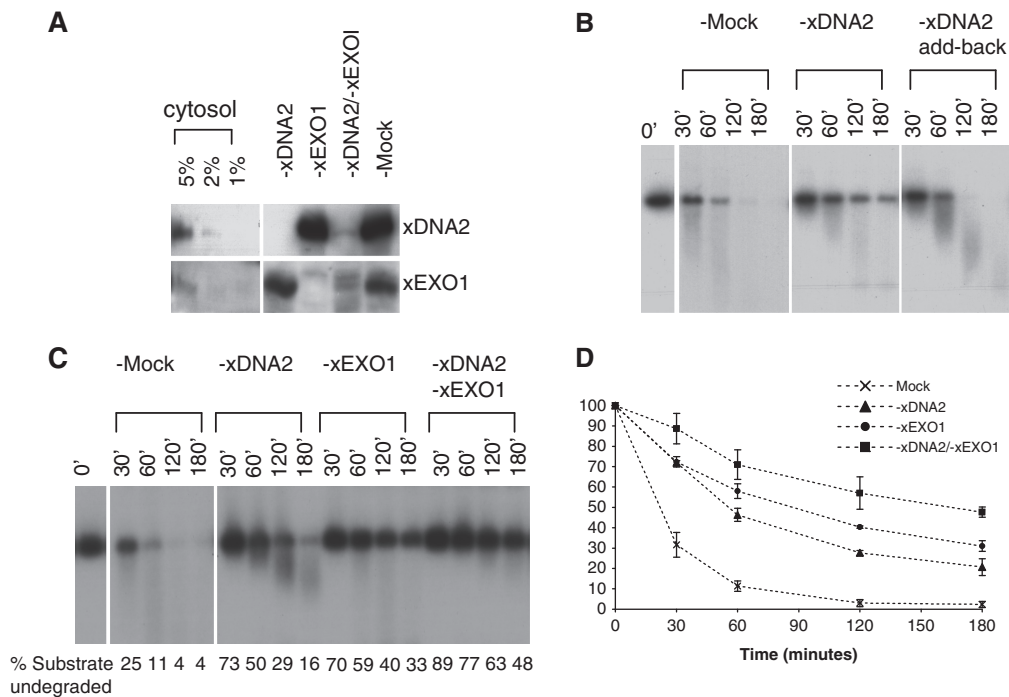


Figure 4. Effect of xEXO1 and xDNA2 co-depletion on 5'-strand resection. (A) Efficiency of xEXO1 and xDNA2 depletion. The quantitation standards for western blot were untreated cytosol at different amounts relative the depleted cytosol. (B) Effect of xDNA2 single depletion on 5'-strand processing in cytosol. The 3'- ^{32}P -labeled linear pBLP DNA with ddC-blocked ends (1 ng/ μl) was incubated in mock-depleted cytosol or xDNA2-depleted cytosol (supplemented with either the ELB buffer or the purified xDNA2). Samples were taken at the indicated times, treated with SDS-proteinase K and analyzed on a 1% TAE-agarose gel. (C) Effect of xEXO1 and xDNA2 double depletion on 5'-strand processing. Cytosol depleted of the indicated proteins was incubated with the 3'-labeled ddC-blocked linear pBLP (1 ng/ μl). Samples taken at the indicated times were treated with SDS-Proteinase K, separated on a 1% TAE-agarose gel, transferred to a Nylon membrane, and detected by Southern hybridization with a ^{32}P -labeled probe. (D) Plot of the substrate remaining at the indicated times after incubation in the various depleted extract. The averages and standard deviations were derived from three sets of data.

executes 5'-strand resection indirectly by degrading the 5' ss-tail generated by xWRN at DSBs (4). The purified xEXO1 displayed 5'→3' ds- but not ssDNA exonuclease activity even in the presence of RPA. This activity spectrum suggests that xEXO1 might be different from xDNA2 and act directly on ds-DNA during 5'-strand resection. However, there might be a factor (other than RPA) in cytosol that could stimulate the ss-exonuclease activity of xEXO1 and consequently xEXO1 might also act on ssDNA intermediates. To test this idea, we determined the effect of xEXO1 depletion on the degradation of linear ssDNA, which mimics the intermediates formed by helicase-mediated end unwinding. As shown in Figure 5, ssDNA, which migrated as smears, was rapidly degraded in mock-depleted cytosol but very stable in xDNA2-depleted cytosol, which was consistent with previous observations in NPE (4). In contrast to xDNA2 depletion, xEXO1 depletion did not cause any significant effect on the degradation of ssDNA. Double depletion of xEXO1 and xDNA2 was also very similar to xDNA2 single depletion in degrading linear ssDNA. Taken together, these data suggested that xEXO1, in contrast to xDNA2, executes 5'-strand resection by acting directly on ds-DNA rather than indirectly on 5'-ssDNA.

xEXO1 acts in parallel to xWRN

To further define the mechanistic role of xEXO1 in 5'-strand resection, we next analyzed its epistatic relationship to xWRN, the major RecQ helicase that acts upstream of xDNA2 in the *Xenopus* NPE system (3,4). It has been reported that hEXO1 is stimulated by WRN in a manner independent of ATP and helicase activity (14). Therefore, xEXO1, while not acting on the ss-DNA generated by xWRN, might still act in conjunction with xWRN through physical stimulation of xEXO1's dsDNA exonuclease activity. To test this hypothesis, we first determined if xWRN is important for 5'-strand resection

by depleting it from cytosol (Figure 6A). As shown in Figure 6B, depletion of xWRN indeed caused a significant inhibition of 5'-strand resection. The inhibition was reversed by the addition of the purified xWRN, indicating that it was specific. We next determined the effect of co-depletion of xWRN and xEXO1 or xDNA2 on 5'-strand resection (Figure 6A and C). When xWRN was co-depleted with xEXO1, 5'-strand resection was more inhibited (2–2.4 fold) than in singly depleted cytosol, with over 65% (doubly depleted) versus 27% (xWRN depleted) and 32% (xEXO1 depleted) of the DNA still undegraded even after 3 h. In contrast, co-depletion of xWRN and xDNA2 was not significantly different from single depletions of either protein. Taken together, these data suggest that xEXO1 acts in a parallel pathway to xWRN, while xDNA2, as expected, acts in the same pathway as xWRN.

xEXO1 and xWRN participate in 5'-strand resection from the beginning

EXO1 is fully active toward ds-DNA, yet genetic analyses in *S. cerevisiae* have suggested a two-stage model for 5' resection: MRX and Sae2 degrade the first few hundred nucleotides and then EXO1 and SGS1/DNA2 take over to carry out more extensive degradation (8,9). It is unclear how MRX and Sae2 actually accomplish the initial degradation of the 5' strand. Complicating the issue is that the limited 5'-resection observed in the *exo1sgs1* double mutant might be the result of leakiness or compensating mutations in the mutant strain. There is also the potential complication that when replication forks collide with DSBs, the lagging strand is expected to carry some 3' ssDNA. The *Xenopus* system is not affected by these potential indirect effects. To determine if xEXO1 and xWRN are required from the very beginning of 5'-strand resection, we analyzed the fate of a 5'-³²P-labeled DNA. End processing proceeds in the 5'→3' direction, so this 5' label would be the first to be removed (Figure 1C).

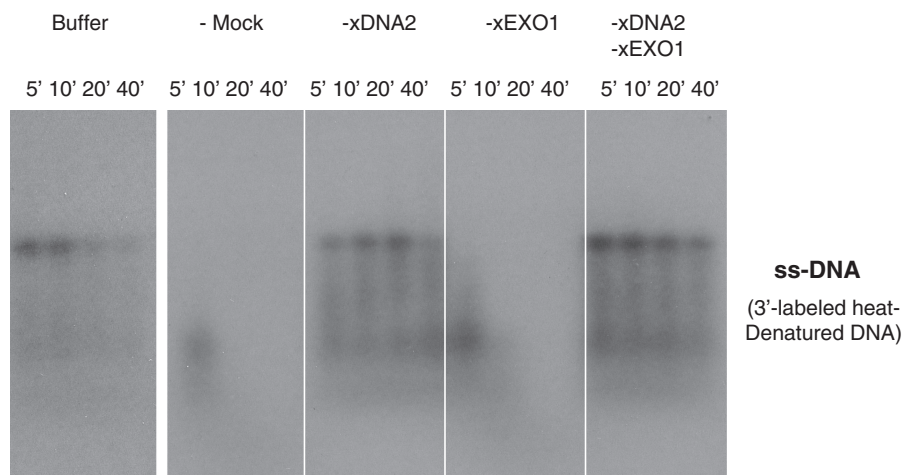


Figure 5. Effect of xEXO1 and xDNA2 on 3'-labeled ssDNA degradation. Linear ss-pBS DNA (2 ng/μl) labeled with ³²P at the 3'-end was incubated in the indicated depleted cytosol or buffer. Samples taken at the indicated times were treated with SDS-proteinase K, separated on a 1% TAE-agarose gel, and detected by exposure to an X-ray film.

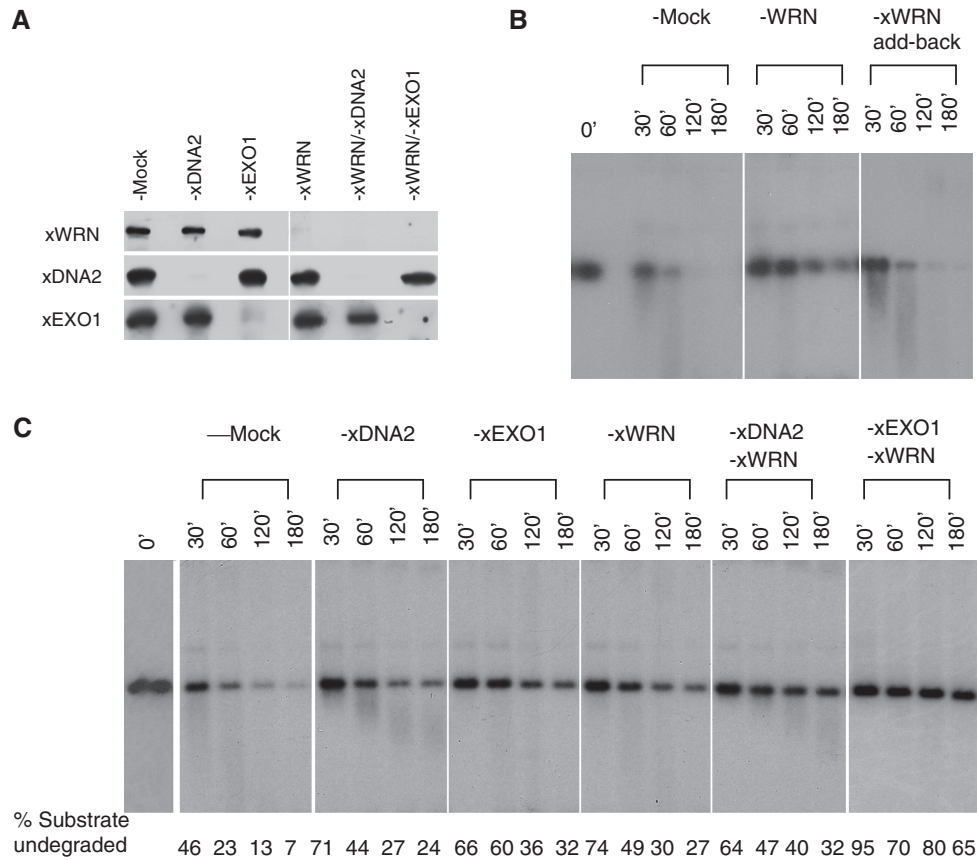


Figure 6. Effect of xEXO1 and xWRN co-depletion on 5'-strand processing. (A) Western showing the depletion of xEXO1, xWRN, and xDNA2. (B) Effect of xWRN single depletion on 5'-strand processing. The 3'-³²P-labeled linear pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated in mock-depleted cytosol or xWRN-depleted cytosol (supplemented with either buffer or the purified xWRN). Time points were treated with SDS-proteinase K and analyzed on a 1% TAE-agarose gel. (C) Effect of xEXO1-xWRN and xDNA2-xWRN double depletions on 5'-strand processing. Cytosol depleted of the indicated proteins were incubated with the 3'-³²P-labeled and ddC-blocked linear pBLP (1 ng/μl). The reactions were treated and analyzed similarly to that in (B). The percentage of remaining DNA substrate was calculated relative to the total input DNA.

As shown in Figure 7, in mock-depleted cytosol, the 5'-³²P was as expected rapidly lost (not retained on the intermediates). However, in cytosol depleted of xEXO1 and xWRN, alone or together, the ³²P label was still retained on the remaining DNA substrate. These results suggest that xEXO1 and xWRN participate in both the initiation stage and the extension stage of resection.

DISCUSSION

EXO1 is a key player in the degradation of 5'-strand DNA at DSBs in yeast *S. cerevisiae* and human cells, yet its mechanism of action has not been rigorously determined. Yeast and human EXO1 display strong 5'→3' exonuclease activity toward dsDNA and weak 5'→3' ss-exonuclease activity toward ssDNA. The ssDNA exonuclease activity was assayed in the absence of the ssDNA binding protein RPA, which is known to strongly stimulate the 5'→3' ssDNA exonuclease activity of DNA2 (4). It thus is possible that EXO1 might be like DNA2 in acting on ssDNA generated by helicase unwinding of ends.

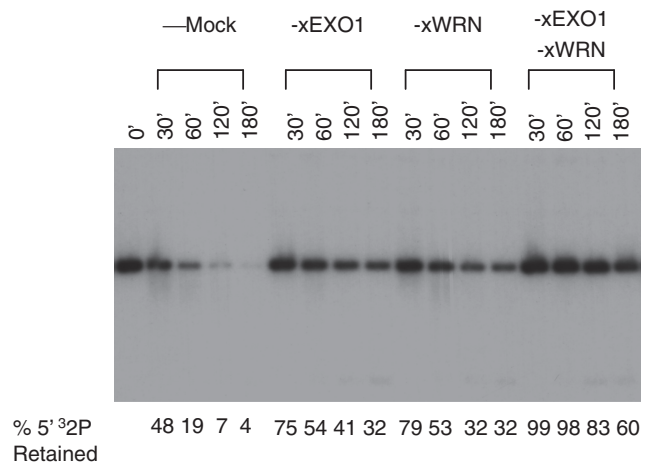


Figure 7. Effect of xEXO1 and xWRN on the initiation of 5'-strand processing. The 5'-³²P-labeled linear pBLP DNA with ddC-blocked ends (1 ng/μl) was incubated with cytosol depleted of the indicated proteins. Samples were taken at the indicated times, treated with SDS-proteinase K, and analyzed by a 1% TAE-agarose gel. The percentage of 5'-³²P-label retained on the substrate was calculated relative to the total input.

Surprisingly, while EXO1 can degrade dsDNA by itself, genetic analyses in *S. cerevisiae* suggest that it affects only the later stage of 5'-strand resection (8,9). In this study, we used a combination of biochemical reconstitution and enzymatic characterizations to investigate the mechanism by which EXO1 executes 5'-strand resection at DSBs. The results have led to a more definitive answer to this important question.

We first established that 5'-resection can be reconstituted in regular cytosol derived from unfertilized interphase *Xenopus* eggs. It has previously been observed that *Xenopus* eggs contain activities that can catalyze 5'→3' strand resection and SSA repair (28). Extracts derived from activated eggs can also catalyze the MRE11 and CtIP-dependent binding of RPA to DNA beads or chromatin (an indirect readout of 5' resection) (21,22). The egg extract we used was prepared essentially in the same way, but the assay detects directly the fate of linear DNA. By placing a ³²P label at either the 5'- or 3'-end, the directionality of DNA resection was easily determined to be 5'→3'. The assay is quantitative, easy to perform (without the need to wash beads or chromatin pellets), and highly scalable to handle multiple reactions and time points. The data presented in this study demonstrated that resection is mediated by xWRN, xDNA2 and xEXO1. We also found that it is inhibited by depletions of xMRE11 and xCtIP (data not shown). Together, these observations strongly suggest that the mechanism for 5'-strand resection is faithfully recapitulated by the egg extract system. Compared to the NPE system we previously used to study resection, cytosol is much easier to prepare. In addition, repair and replication proteins are much less concentrated in cytosol than in NPE, making immunodepletion much easier to perform. This is particularly useful when two or more proteins have to be depleted to analyze their epistatic relationship. With NPE, co-depletion often requires more antibody than the capacity of the protein A beads allows. We expect the cytosol system to be a significant addition to the field of DSB repair research.

By taking advantage of the cytosol system and through enzymatic characterization of purified xEXO1, we found that (i) xEXO1 possesses strong 5'→3' dsDNA exonuclease activity but no significant ssDNA exonuclease activity even in the presence of RPA; (ii) depletion of xEXO1 indeed caused a significant inhibition of 5' strand resection; (iii) xEXO1 and xDNA2 act in parallel pathways; (iv) xEXO1, unlike xDNA2, does not affect the degradation of ssDNA; (v) xEXO1 acts in parallel to xWRN, while xDNA2 acts in the same pathway as xWRN; and (vi) xEXO1 and xWRN are required from the very beginning of 5'-strand resection. The function of EXO1 in 5' resection and its 5'→3' dsDNA exonuclease activity are thus highly conserved in *S. cerevisiae*, *Xenopus* and human cells. However, we did not detect significant 5'→3' ssDNA exonuclease activity, even in the presence of RPA. Consistent with this observation, depletion of xEXO1 had no effect on the degradation of ssDNA in *Xenopus* cytosol. This suggests that even in the context of cytosol, which should contain all the co-factors for xEXO1 (should they exist), xEXO1 cannot degrade ssDNA. It has also been reported that hWRN stimulates the flap

endonuclease activity of hEXO1 (14). However, in the presence of ATP, a flap that is being unwound by WRN is not accessible to EXO1 (14). As such, EXO1 and WRN cannot act together in this way to degrade 5' strand DNA efficiently and processively. Consistent with this idea, we found that the EXO1 pathway remains robust in xWRN-depleted cytosol. Taken together, our data suggest strongly that EXO1 executes 5'-strand resection by acting directly on ds-DNA.

One surprising finding is that xEXO1 and xWRN are required from the very beginning of 5'-strand resection. In cytosol depleted of both xEXO1 and xWRN, the majority of DNA was not degraded and, importantly, still retained the 5'-³²P label. This is in direct contrast to what has been reported in yeast where *exo1 sgs1* double mutant still showed residual 5' resection of a few hundred nucleotides. There are three potential explanations for this difference. The first explanation is that yeast MRX and SAE2, but not *Xenopus* MRN and CtIP, are by themselves capable of limited 5' resection even in the absence of EXO1, SGS1 or DNA2. However, the known enzymatic activities of MRX and SAE2, which have been reported to possess 3'→5' exonuclease activities, do not provide clues to how this might be achieved. A mutation that inactivates the endonuclease activity of MRE11 has no effect on 5'-strand resection or homologous recombinational repair (29). The second explanation is that the limited 5'-strand resection in the *exo1 sgs1* double mutant is the result of DNA replication, which is expected to leave behind some 3'-ssDNA on the lagging strand. The third explanation is that since EXO1, SGS1 and DNA2 are all important for genome maintenance, the yeast mutants inactivating these genes might have acquired compensating mutations to survive and thrive. Indeed, there are many nucleases in yeast and their functions are often overlapping, especially when over-expressed (30). Genetic analysis has shown that EXO1 and RAD27, which encodes a flap endonuclease, have overlapping functions in DSB repair (31). While *exo1* or *rad27* single mutants are viable, the *exo1 rad27* double mutant is inviable in some genetic background (32) but viable in another, implying overlapping functions with even more proteins (33). Similarly, the lethality of *sgs1 srs2* double mutant can be suppressed by inactivating genes involved in homologous recombination (34). Finally, to study the role of DNA2 in end resection, the *dna2Δ* mutation, which is lethal, has to be suppressed by *pif1-m2* (9). Based on these considerations, it is possible that the *exo1 sgs1* or *dna2* mutant strains have acquired some compensating activity to carry out limited 5'-strand resection. Compared to yeast analysis, the *Xenopus* system does not suffer from these potential indirect effects of mutant strains. Notably, in four 5'-resection systems reconstituted with purified proteins, DNA degradation is consistently shown to be executed by DNA2 and EXO1, while MRX/MRN promotes resection and SAE2/CtIP is either stimulatory or completely dispensable (35–38). Taken all these observations together, it is reasonable to conclude that xEXO1 and xWRN-xDNA2-RPA are required for not only the extension stage but also the initiation stage of resection. However, we cannot rule out the possibility that there are yet to be identified activities of

MRE1 and SAE2/CtIP or novel nucleases that mediate the initial resection in yeast but not in our system and not required for the purified proteins.

Integrating the results from the current study and other studies in various model systems, we propose that 5'-strand resection is executed by two mechanistically distinct pathways that branch from the beginning (Figure 8). One pathway is catalyzed by EXO1, which acts directly on ds-DNA. The other pathway is catalyzed by WRN (or another RecQ-type helicase), which unwinds ends, and DNA2, which degrades the 5' ss-tail. Both steps of the WRN-DNA2 pathway are stimulated by RPA. RPA also coats the 3'-ssDNA generated by the two pathways and protects it from non-specific degradation. Both pathways require MRN complex and CtIP to initiate, but EXO1 and WRN helicases are also essential components at this step. MRN and CtIP are later released after the 5'-strand is partially degraded. The observations in this study and those with purified proteins suggest that EXO1 and DNA2 are the nucleases that degrade DNA, while MRN and CtIP promote their recruitment to or activity at DNA ends. Further studies will be needed to test this model and elucidate the exact mechanistic roles of the respective proteins in the reactions.

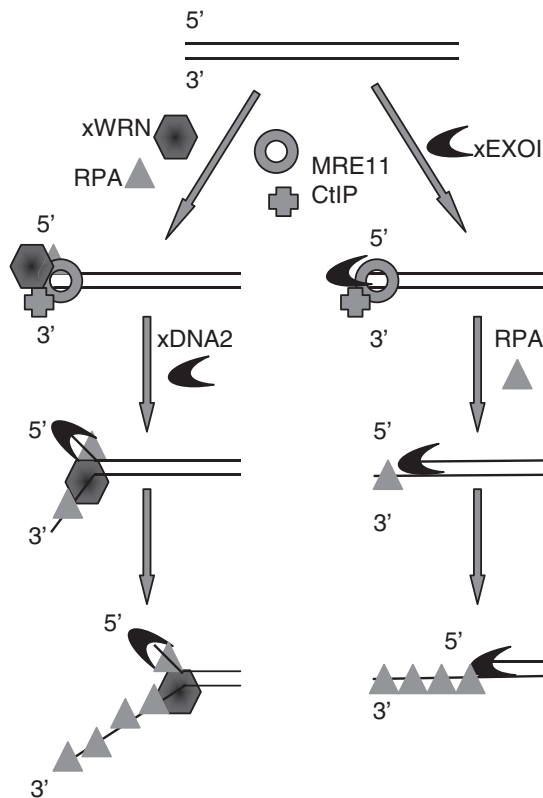


Figure 8. Model for the 5'-strand-specific processing of DNA ends. xWRN-xDNA2 and xEXO1 constitute two parallel pathways. MRN and CtIP act with xWRN-xDNA2 and xEXO1 in the initiation step, but are dispensable after partial degradation of the 5'-strand.

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