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Identification of the *Xenopus* DNA2 protein as a major nuclease for the 5′→3′ strand-specific processing of DNA ends

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ABSTRACT

The first step of homology-dependent DNA double-strand break (DSB) repair is the 5′ strand-specific processing of DNA ends to generate 3′ single-strand tails. Despite extensive effort, the nuclease(s) that is directly responsible for the resection of 5′ strands in eukaryotic cells remains elusive. Using nucleoplasmic extracts (NPE) derived from the eggs of *Xenopus laevis* as the model system, we have found that DNA processing consists of at least two steps: an ATP-dependent unwinding of ends and an ATP-independent 5′→3′ degradation of single-strand tails. The unwinding step is catalyzed by DNA helicases, the major one of which is the *Xenopus* Werner syndrome protein (xWRN), a member of the RecQ helicase family. In this study, we report the purification and identification of the *Xenopus* DNA2 (xDNA2) as one of the nucleases responsible for the 5′→3′ degradation of single-strand tails. Immunodepletion of xDNA2 resulted in a significant reduction in end processing and homology-dependent DSB repair. These results provide strong evidence that xDNA2 is a major nuclease for the resection of DNA ends for homology-dependent DSB repair in eukaryotes.

INTRODUCTION

Among the numerous types of DNA damages that a cell encounters, DNA double-strand breaks (DSBs) are perhaps the most deleterious. If not repaired or improperly repaired, DSBs would lead to chromosome deletions and translocations, causing premature cell death or oncogenic transformation. In eukaryotes, three major pathways have been identified to repair DSBs: non-homologous end joining (NHEJ), homologous recombination (HR) and single-strand annealing (SSA) (1–3). In NHEJ, DNA ends are ligated either directly (for compatible or blunt ends) or after some limited fill-in/degradation (for incompatible ends). In HR, DNA ends are processed into extensive 3′ single-stranded (ss-) tails, which then invade a homologous sequence to copy the missing information. SSA is often used to repair a break that occurs between two direct repeats, which are common in the genome of higher eukaryotes. DNA ends are also processed into 3′ ss-tails, but the two tails on each side of the break anneal with each other, leading effectively to the deletion of one of the two repeats and the intervening sequence.

The first step of HR and SSA is the processing of DSBs into 3′ ss-tails. In *Escherichia coli*, the RecC nuclease in the RecBCD complex is a major nuclease for degrading the 5′ strand after the ends are unwound by the RecB and the RecD helicases (4). In addition, the RecJ 5′→3′ ssDNA exonuclease has been proposed to degrade the 5′ strand following end unwinding mediated by RecQ (5). In eukaryotes, the nuclease responsible for the strand-specific degradation of DNA ends has yet to be definitively identified. Genetic analyses in the budding yeast *Saccharomyces cerevisiae* suggest that the MRE11-RAD50-XRS2 (MRX) complex (MRE11-RAD50-NBS1 or MRN in higher eukaryotes) plays an important role in end processing (6). MRE11 has both an exonuclease and an endonuclease activity (7–10), but the directionality of the exonuclease activity is 3′→5′ rather than 5′→3′. Furthermore, a nuclease-inactivating mutant that can still form the MRX complex shows no significant defect in end processing, whereas other mutations that block end processing appear to do so by destabilizing the MRX complex (11–13). Mre11 interacts with Sae2 (Ctp1 in *Schizosaccharomyces pombe* and CtIP in higher eukaryotes), and disruption of Sae2/CtIP blocks end processing (14–16). Sae2 has an endonuclease activity on DNA structures like hairpins, but how this activity contributes to the 5′→3′ resection is unclear (17). Another candidate nuclease is EXO1, which, when overexpressed, can suppress the mitotic DNA repair defect of *mre11*, *rad50* and *xrs2* mutants (18). While EXOI has a 5′→3′ exonuclease activity, an *exoI* null mutant has no significant defect in recombinational repair (19,20) and only minor defect in DNA end processing (12).

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Therefore, if EXOI is directly involved in end processing, it must do so in redundancy with other 5′→3′ exonucleases.

We have used the *Xenopus* nucleoplasmic extract (NPE) as the model system to study DSB repair and end processing. We found that this system can efficiently reconstitute both NHEJ and SSA. This allowed us to show by immunodepletion that the *Xenopus* Werner syndrome protein (xWRN) plays an important role in SSA (21). Further analysis of xWRN's role in SSA led to the elucidation of a mechanism for the 5′ strand-specific processing of DNA ends (22). End processing in NPE consists of two steps: first the end is unwound and then the 5′ ss-tail is specifically degraded, resulting in the 3′ ss-tail as the final product. This mechanism is remarkably similar to the model that has been proposed for the *E. coli* RecQ helicase and the RecJ 5′→3′ ssDNA exonuclease. While xWRN is one of the eukaryotic homologs of RecQ, database search did not reveal the existence of an eukaryotic homolog of RecJ. We thus used biochemical methods to purify and identify the 5′→3′ ssDNA exonuclease in *Xenopus* extracts. In this study, we show that DNA2 (xDNA2) is one of the major 5′→3′ exonucleases in *Xenopus* extracts. We also show that depletion of xDNA2 from NPE results in a significant reduction in DNA end processing and this defect can be complemented by the purified xDNA2 protein. Corresponding to the defect in end processing, the efficiency of the SSA repair pathway is reduced. Together, these results provide stsrong evidence that xDNA2 is a major nuclease for the strand-specific processing of DNA ends and the subsequent homology-dependent DSB repair.

MATERIALS AND METHODS

Extract preparation

Crude extracts, membrane-free cytosol and nucleoplasmic extracts were prepared from unfertilized *Xenopus* eggs following the standard protocols (23,24).

Nuclease purification

Approximately 10 ml membrane-free cytosol was diluted with equal volume of buffer ELB [10 mM HEPES (pH 7.5), 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol (DTT)] and then fractionated by ammonium sulfate at 37.5–50% saturation (25). The pellet was dissolved in buffer A0 (25 mM Tris.HCl/1mM EDTA/10% glycerol), adjusted to 100 mM conductivity with A1000 (A0 + 1M NaCl), and fractionated on a 5 ml Q Sepharose column (GE Healthcare, NJ, USA) with 100 ml of buffer delivered in a gradient from A100 (A0 + 100 mM NaCl) to A500 (A0 + 500 mM NaCl). The active fractions (peak at 250 mM NaCl) were pooled and directly loaded onto a 2 ml Heparin Sepharose column (GE Healthcare) and eluted with a 40 ml gradient from A200 (A0 + 200 mM NaCl) to A650 (A0 + 650 mM NaCl). The active fractions (peak at 380 mM NaCl) were dialyzed against 50 volumes of buffer H5 [5 mM K_iPO_4 (pH 7.5)/ 10% glycerol/1 mM DTT] and loaded on a 1 ml Hydroxyapatite column (BioRad, CA, USA) and eluted with a 20 ml gradient from K5 to K500 (500 mM K_iPO_4/ 10% glycerol/1 mM DTT). The active fractions (peak at 190 mM of K_iPO_4) were concentrated with Amicon Ultra-4 (Millipore, MA, USA) down to 0.5 ml and fractionated on a 24 ml Superdex 200 gel filtration column (GE Healthcare) with buffer A100. The active fractions (peak at 9 ml elution volume) were concentrated with Amicon Ultra-4 down to 100 µl and then incubated with 33 µl of Streptavidin magnetic beads (Invitrogen, CA, USA) precoated with 33 pmol of a 48-mer ss-oligonucleotide with a biotin at the 3′-end. After 1 h of incubation at 4°C, the beads were washed twice with 0.5 ml A100, once with 50 µl A100, and finally eluted with 40 µl A1000 (A0 + 1 M NaCl).

Mass spectrometry

The proteins in the oligonucleotide beads elute were separated on a 4–12% SDS–PAGE and visualized by Coomassie blue staining. The selected gel bands were excised and destained with 50% MeOH/5% HOAC in water for overnight and then dehydrated completely with 100% acetonitrile. Reduction and alykylation were performed with 20 mM DTT and 50 mM iodoacetamide (IAA). After a second dehydration, the gel bands were rehydrated at 4°C for 45 min in trypsin solution [10 ng/µl sequencing grade modified trypsin (Promega, WI, USA)/ 10 mM NH_4HCO_3/5% acetonitrile]. Proteins were digested at 34°C for 4 h. For MALDI-TOF peptide mass fingerprinting, 0.3 µl sample and 0.3 µl matrix (CHCA) were spotted onto MALDI target plate and allowed to dry. Mass spectra were acquired with a Reflex IV mass spectrometer (Bruker Daltonics, Billerica, MA, USA) between 500 and 5000 m/z in reflectron mode and peptide peaks were calibrated internally using trypsin autolysis peaks. Proteins were identified using automated MASCOT (www.matrixscience.com) and BioTool (Bruker Daltonics) software, and further analyzed through database searches (SwissProt, BLAST, NCBI, and MSDB).

Nuclease assay

The substrates for the nuclease assay were prepared as previously described (22). ^{32}P-labeled biotinylated 48-mer oligonucleotides, in either the single-stranded form or the double-stranded form, were coated onto Streptavidin paramagnetic beads following the manufacturer's instruction (Invitrogen). The substrates were designated as 5′ or 3′ depending on the accessible end on the ^{32}P-labeled strand after attachment to beads. A typical nuclease reaction contained 5 µl protein to be assayed (in A250 buffer or equivalent buffer), 5 µl ELB buffer, and 0.1 µl of oligonucleotide beads (0.5 ng DNA/µl beads). After incubation at room temperature for 30–60 min with rotation, the reactions were terminated with 1% SDS, heated at 95°C for 10 min and analyzed by 10% TAE/PAGE.

DNA end processing assays and unwinding assays

End processing assays and unwinding assays were performed essentially as previously described (22). The DNA substrate for end processing was prepared by digesting

pUC19 or pBS DNA with BamHI and filling the ends with dGTP and ^{32}P dATP. Single-stranded DNA was prepared by denaturing the labeled pUC19 by heat and then immediately chilling it on ice. A typical DNA end processing assay contained: 5 µl depleted NPE, 0.5 µl 10× ATP mix (20 mM ATP/200 mM phosphocreatine/0.5 mg/ml creative kinase/50 mM DTT), 0.375 µl DNA (40 ng/µl), 0.75 µl 2.5 mM ddNTPs, 0.875 µl xDNA2 protein or ELB buffer. The reactions were incubated at room temperature, 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 µl with H_2O and supplemented with 1 µl 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 Phosphoimager (Fuji) to detect ^{32}P.

The substrate for unwinding was a 48-mer double-stranded oligonucleotide with one strand carrying a biotin at the 5′ end and the complementary strand containing 21 thionucleotides (incorporated by Klenow) on the 3′ half and ^{32}P-label at the 3′-end. The DNA was coated onto Streptavidin magnetic beads (Invitrogen) following the standard procedure. A typical unwinding reaction contained: 5 µl depleted NPE, 0.5 µl 10× ATP mix, 0.5 µl DNA beads (0.5 ng/µl), and 1.5 µl ELB buffer. After incubation at room temperature with rotation, 3.75 µl of each reaction was withdrawn at the indicated time and mixed with 11.25 µl washing buffer (10 mM Tris HCl (pH 8)/1 mM EDTA/1 M NaCl/0.05% NP-40). The beads were isolated by magnet (10 µl of the supernatants were saved), washed with 15 µl washing buffer and resuspended in 10 µl washing buffer. The supernatant and bead fractions were mixed with 3.3 µl 4% SDS/50 mM EDTA, 6.7 µl H2O and 2 µl Proteinase K (10 mg/ml). After incubation at room temperature for 3 h, samples were analyzed on a 10% TAE/PAGE.

SSA assay

The SSA was assay was performed essentially as previously described (21). The substrate was plasmid pRW4 linearized by XhoI, which cut between two 1.2 kb direct repeats, and then partially filled-in with TTP and dCTP. A typical SSA assay contained 0.5 µl 10× ATP mix, 5 µl xDNA2-depleted (supplemented with the purified xDNA2 or ELB) or mock-depleted NPE, and 20 ng/µl DNA in a 7.5 µl reaction. After incubation at room temperature, 1.8 µl samples were taken at the indicated times and mixed with 1.8 µl 2% SDS/25 mM EDTA, 6.4 µl H_2O, and 1 µl Proteinase K. After incubation at room temperature for 6 h, the DNA samples were separated on 1% TAE/agarose gels and detected by SYBR Gold (Invitrogen).

Antibody preparation and immunodepletion of xDNA2

The cDNA encoding the N-terminal 712 amino acids of xDNA2 was isolated by PCR and subcloned into a pGEX expression vector. The GST–xDNA2 fusion protein was expressed in BL21(DE3), isolated from a SDS–PAGE gel, and injected into two rabbits. The anti-xDNA2 antibodies were purified with two affinity columns constructed respectively with the GST–xDNA2 fusion protein and the GST affinity tag following a protocol published previously (26). To deplete xDNA2, NPE (40 µl + 20 µl ELB) was incubated at 4°C for 2.5 h with 20 µl Protein A Sepharose beads precoated with 7.5 µg of the affinity-purified rabbit anti-xDNA2 antibodies or buffer. The procedure was repeated and the depleted NPE was saved as 5 µl aliquots at –80°C.

RESULTS

Purification and identification of a 5′→3′ ssDNA exonuclease

The xWRN-mediated end processing pathway is remarkably similar to the model that has been proposed for the *E. coli* RecQ helicase and the RecJ 5′→3′ ssDNA exonuclease. While xWRN is one of the eukaryotic homologs of RecQ, our database search did not reveal the existence of an eukaryotic homolog of RecJ. We thus used biochemical methods to purify and identify the 5′→3′ ssDNA exonuclease in *Xenopus* egg extracts (Figure 1A). The *Xenopus* extract contains efficient 5′→3′ ss-exonuclease activity but no significant 3′→5′ ss-exonuclease activity or endonuclease activity (22). By biochemical fractionation, the major 5′→3′ ssDNA exonuclease activity could be separated into two fractions, one with a low level of nuclease activity and one with a stimulating factor, by heparin column. The nuclease assay used a 48-mer ss-oligonucleotide with two ^{32}P dA and a biotinylated dC at the 3′-end as the substrate. After binding to Streptavidin magnetic beads, the 3′ end was blocked but the 5′ end accessible. The 250 mM NaCl elute, but not the flow through, contained a low nuclease activity. When the two fractions were combined, the nuclease activity was greatly stimulated (Figure 1B). The nuclease fraction was further purified by hydroxyappatite and gel filtration. For the final step of purification, the active fraction from the gel filtration column was incubated with 5′ ss-oligonucleotides (unlabeled) attached to magnetic beads. As shown in Figure 1C, a major band of ~120 kD could efficiently bind to DNA beads and then be eluted with a buffer containing 1 M NaCl. Correspondingly, the 5′→3′ exonuclease activity was depleted from the DNA bead-treated supernatant but recovered in the elute (Figure 1D, lanes 1–3). These data strongly suggested that the 120 kD protein was responsible for the 5′→3′ ssDNA nuclease activity. Mass spectrometry analysis of this protein excised from an SDS–PAGE identified it as the *Xenopus* homolog of the budding yeast DNA2 (xDNA2). (The faint band just below was a partial degradation product of xDNA2.) Consistent with this identification, the sequence of xDNA2 contains a RecC-type nuclease domain (27).

Identification of the stimulatory factor for xDNA2

We also identified the stimulating factor for xDNA2 as xRPA based on three lines of evidence. Western analysis showed that the heparin column flow through contained xRPA (Figure 2A). As expected, this xRPA could be removed by incubation with 5′ ss-oligonucleotide beads.

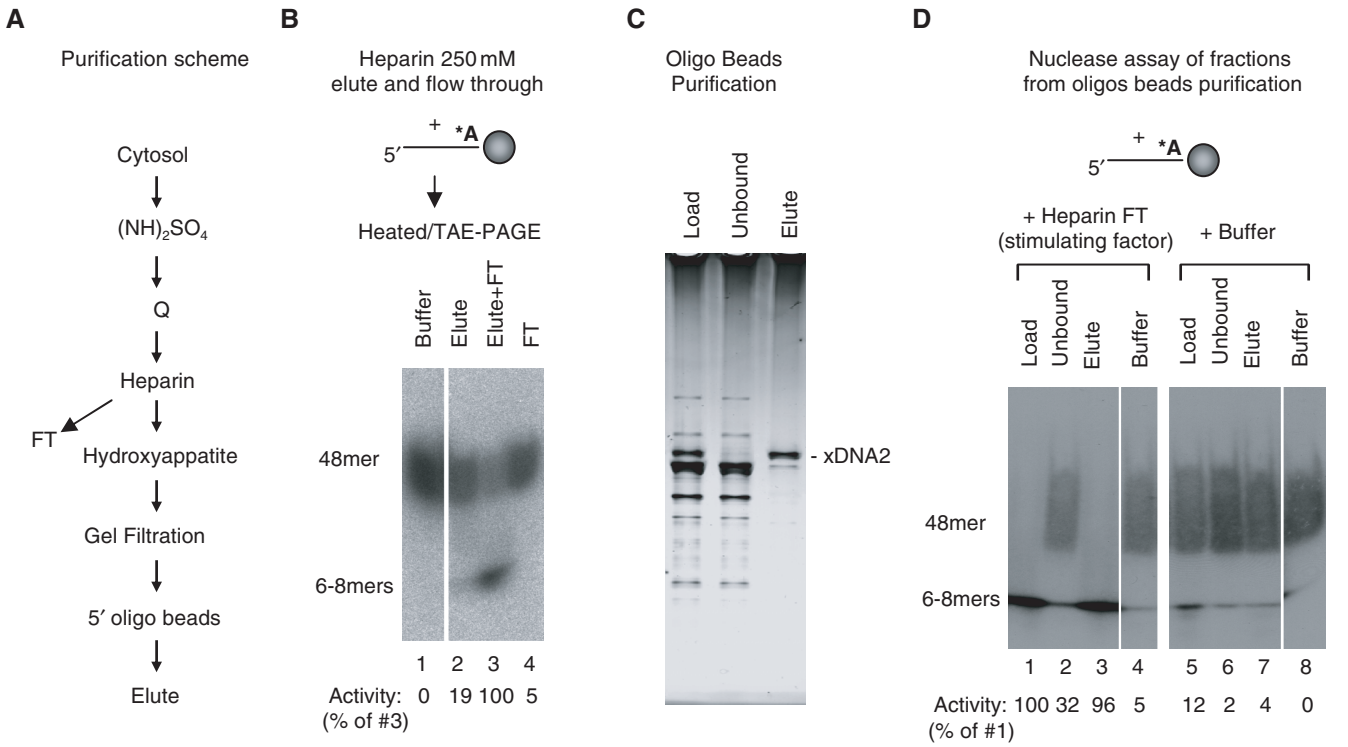

Figure 1. Purification and identification of the major 5′→3′ ssDNA exonuclease in *Xenopus* extracts. (**A**) Purification scheme. (**B**) Nuclease assay of the heparin flow through and 250 mM NaCl elute. The oligo substrate carried two ^{32}P-labeled dA near the 3′-end and was attached to magnetic beads through a biotin at the 3′-end. After incubation at room temperature for 1 h, the reactions were terminated with 1% SDS, heated at 95°C for 5 min and then analyzed on an 8% TAE/PAGE. The ^{32}P signal was detected by Phophoimager (Fuji). The nuclease activity was determined by calculating the fraction of small product generated (normalized to that of reaction 3 that contained both the 250 mM elute and the flow through). (**C**) A silver stained SDS–PAGE gel (4–12%) of the purified xDNA2 after the oligo beads purification. Load, proteins before binding to the oligo beads; unbound, proteins after incubation with beads; elute, bound proteins eluted by high salt buffer. (**D**) Nuclease assay of the purified xDNA2 in the presence of heparin flow through or buffer. The assay condition and the determination of the nuclease activity were similar to those in (**B**).

Concomitantly, the stimulating activity was also removed after incubation with ssDNA beads (Figure 2B, lanes 1 and 2). Most importantly, the purified xRPA could equally stimulate the 5′→3′ ss-exonuclease activity of the purified xDNA2 (Figure 2B, lanes 3 and 4). 30 nM and 90 nM xRPA showed similar stimulatory activity, while xRPA by itself showed barely detectable degradation even at 90 nM (Figure 2B, lane 6). Together, these data suggest that xRPA is the stimulating factor for xDNA2. The stimulatory effect of xRPA was highly specific for the 5′→3′ ssDNA exonuclease activity of xDNA2. As shown in Figure 2C, even in the presence of xRPA, xDNA2 displayed no detectable activity toward 3′ ssDNA or dsDNA. In a previous study, the recombinant *Xenopus* DNA2 displays only a very low level of ssDNA exonuclease activity (27). However, RPA was not used in that study, which probably accounted for the failure to detect the 5′→3′ ssDNA exonuclease activity.

Effect of xDNA2 depletion on DNA end processing

To determine if xDNA2 is indeed involved in DNA end processing, we prepared antibodies against the N-terminal 712 amino acids of xDNA2. The anti-xDNA2 antibodies were purified and then used to deplete xDNA2 from NPE. As shown in Figure 3A, xDNA2 could be removed to a level below detection (>98% depletion). The substrate for DNA end processing was a linear pUC19 DNA that had been labeled with ^{32}P-dATP at the 3′-end by Klenow. NPE can efficiently process this DNA in the 5′→3′ direction if NHEJ pathway is blocked by ddNTPs (22). In mock-depleted NPE, the DNA was rapidly processed, as detected by both SYBR Gold DNA staining and the ^{32}P label (Figure 3B). In contrast, in xDNA2-depleted NPE, end processing was greatly inhibited (Figure 3B, −xDNA2 NPE). Even after 120 min of incubation, there was still a significant amount of partially processed DNA left in xDNA2-depleted NPE. The remaining end processing was probably due to a combination of the residual xDNA2 and the presence of a functionally redundant nuclease. The xDNA2 depletion effect was specific because it could largely be reversed by the addition of the purified xDNA2 protein (Figure 3B, compare the 120′ time points of the −xDNA2 NPE + xDNA2 or buffer). Notably, the xDNA2 protein itself displayed no nuclease activity towards the dsDNA substrate, consistent with its specificity towards ssDNA and dependence on RPA (which was not included in these reactions) (Figure 3B, buffer + xDNA2). Together, these results indicate that xDNA2 is indeed important for DNA end processing.

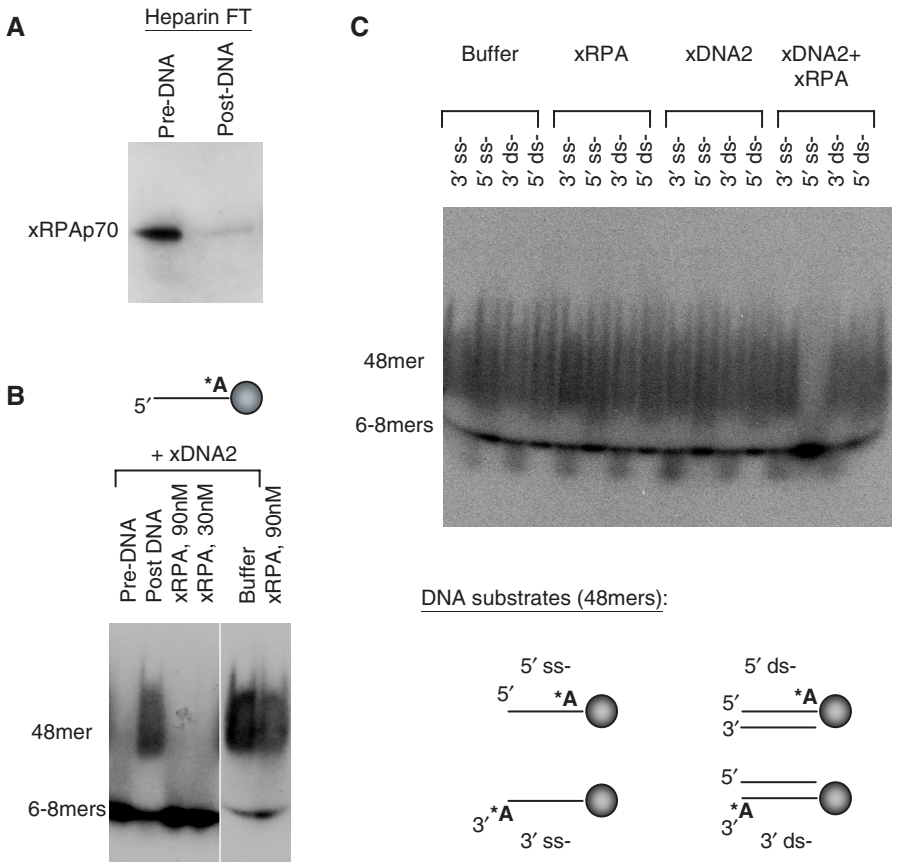

Figure 2. Identification of xRPA as the stimulating factor for xDNA2. (**A**) Western blot of xRPA (p70 subunit) in the heparin flow through before and after incubation with ss-oligo beads. (**B**) The stimulating activity in heparin flow through, before and after incubation with oligo beads, and pure xRPA (at 90 nM and 30 nM). The reactions were terminated with 1% SDS, heated at 95 C for 5 min, and then analyzed on an 8% TAE/PAGE. (**C**) Nuclease assay with different DNA substrates. The substrates were 48-mers in either ss- or ds-form. They were attached to magnetic beads, leaving either the 5′ or the 3′-end (of the ^{32}P-labeled strand) accessible to the nuclease. The assay condition was similar to that in (**B**).

Effect of xDNA2 on single-strand tail degradation

We next investigated the mechanism by which xDNA2 promotes DNA end processing. End processing proceeds in at least two steps: unwinding of the dsDNA end and the degradation of the 5′ ss-tail (22). As a 5′→3′ ssDNA exonuclease activity, xDNA2 was expected to catalyze the 5′ ssDNA degradation. This hypothesis was tested by incubating heat-denatured linear ss-pUC19 DNA in xDNA2-depleted or mock-depleted NPE. As shown in Figure 4, while the ssDNA was rapidly degraded in mock-depleted NPE, it was much more stable in xDNA2-depleted NPE. This effect was specific because when the purified xDNA2 protein (no RPA) was added back to the reaction, the ss-pUC19 DNA was again degraded. [The kinetics was slightly slower than in mock-depleted NPE due to the limited amount of xDNA2 added back (~5% of the endogenous xDNA2).] These results show that xDNA2 is indeed important for ss-tail degradation.

xDNA2 is not important for the unwinding of DNA ends

Yeast DNA2 is both a nuclease and a helicase. While *Xenopus* DNA2 displays no helicase activity *in vitro*, this might be similar to the exonuclease activity in that a stimulatory factor for the helicase activity is missing. We thus examined the potential effect of xDNA2 on DNA end unwinding. The substrate for the unwinding assay was a 48 bp oligonuleotide duplex, with one strand carrying a biotin moiety at its 5′-end and the complementary strand carrying 24 normal nucleotides in the 5′ half followed by 21 thionucleotides (resistant to nuclease digestion) in the 3′ half and ^{32}P-dA near the 3′-end (Figure 5A). The oligonucleotide duplex was first bound onto Streptavidin magnetic beads and then incubated with xDNA2-depleted or mock-depleted NPE. End processing could only proceed from the 5′-end of the thio-strand and then stall at thionucleotides (22). Without unwinding, the partially degraded thio-strand would have remained annealed to the biotin strand and thus bound to the beads. With unwinding, in contrast, it would have been released from the beads. In mock-depleted NPE, the partially degraded thio-strand was as expected unwound and released into the supernatant (Figure 5B). In xDNA2-depleted NPE, the thio-strand was also efficiently released into the supernatant, suggesting that xDNA2 depletion did not affect unwinding. Notably, the released oligo migrated much slower than the oligo released in mock-depleted NPE. This again showed that in the absence of xDNA2, the unwound ssDNA was not efficiently degraded. Together,

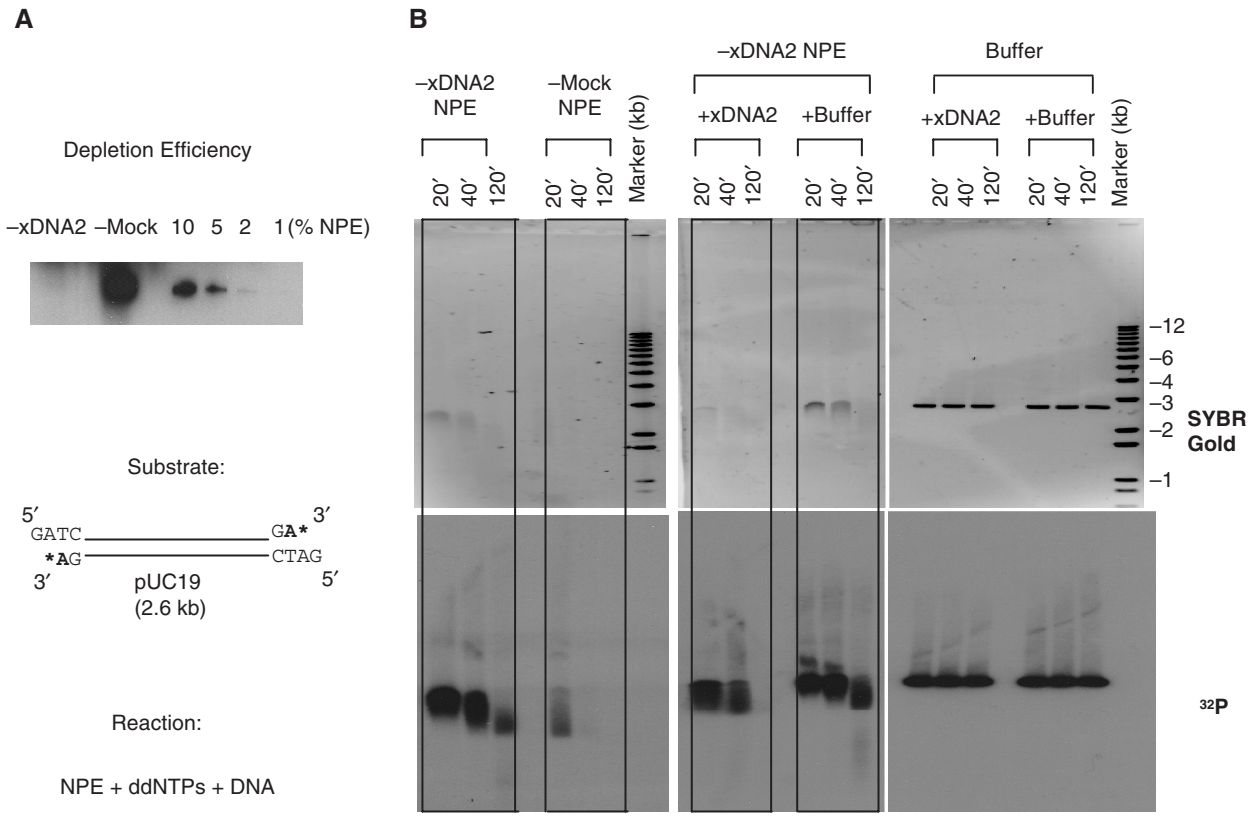

Figure 3. Effect of xDNA2 on DNA end processing. (**A**) Depletion efficiency, end processing substrate, and reaction set up. The quantitation standards for western analysis were NPE loaded at the indicated amounts relative the depleted NPE. The 3′-ends of the partially filled-in linear pUC19 were labeled with ^{32}P-dA by Klenow. (**B**) Effect of xDNA2 depletion on end processing. DNA (2 ng/μl) was incubated in xDNA2-depleted NPE, mock-depleted NPE or xDNA2-depleted NPE supplemented with either ELB buffer or the purified xDNA2 protein (to ~15% of the endogenous xDNA2 level) in the presence of ddNTPs (to block NHEJ). Two additional reactions containing xDNA2 or ELB buffer served as controls. Samples were taken at the indicated time, treated with SDS/proteinase K and separated on a 1% TAE/agarose gel. The gel was first stained with SYBR Gold to detect total DNA and then dried for exposure to X-ray film to detect ^{32}P.

these data indicated that xDNA2 is not required for the unwinding of dsDNA ends.

xDNA2 is important for SSA

Strand-specific end processing is the initiating step for homology-dependent DSB repair. While the above data showed that xDNA2 plays an important role in DNA end processing, it is unclear if this function contributes to DSB repair or is merely for degrading DNA. To address this question, we determined the effect of xDNA2 depletion on SSA, which could be efficiently reconstituted in NPE (21). The substrate for SSA was a 5.6 kb linear DNA with two 1.2 kb direct repeats at the ends. SSA in NPE occurs preferentially intermolecularly, resulting in dimers with only one repeat (as opposed to two repeats by NHEJ) retained at the junction. NHEJ is also active and produces circular monomers (both supercoiled and relaxed) and dimers. Multimers composed of more than two molecules are also produced after longer incubation by continued intermolecular repair. As shown in Figure 6, depletion of xDNA2 caused a dramatic reduction in the formation of SSA repair products. This effect was specific as it could be complemented to a significant extent by the addition of the purified xDNA2 protein (to ~5% of the endogenous level). For NHEJ products, there was no reduction but a slight increase in dimer formation, presumably because more DNA molecules were channeled to this pathway. (The circular monomers migrated mostly as the relaxed form in xDNA-depleted NPE, but this effect was not reproducible.) Together, these data demonstrate that the xDNA2-mediated end processing is coupled to the downstream homology-dependent DSB repair pathway.

DISCUSSION

Previously, we have shown that dsDNA ends are processed by a two-step mechanism (21). The end is first unwound by a helicase, mainly xWRN in NPE, into 5′ and 3′ ssDNA tails in an ATP-dependent reaction. An unknown 5′→3′ ssDNA exonuclease(s) then degrades the 5′ ss-tail in an ATP-independent reaction. The lack of significant 3′→5′ ss- exonuclease activity in NPE ensures that the 3′ ss-tail remains as the final processing product. This mechanism is remarkably similar to the model that has been proposed for the *E. coli* RecQ/RecJ proteins except that the exonuclease corresponding to RecJ is unknown. In this study, we purified and identified xDNA2 as one of the major 5′→3′ ssDNA exonucleases in

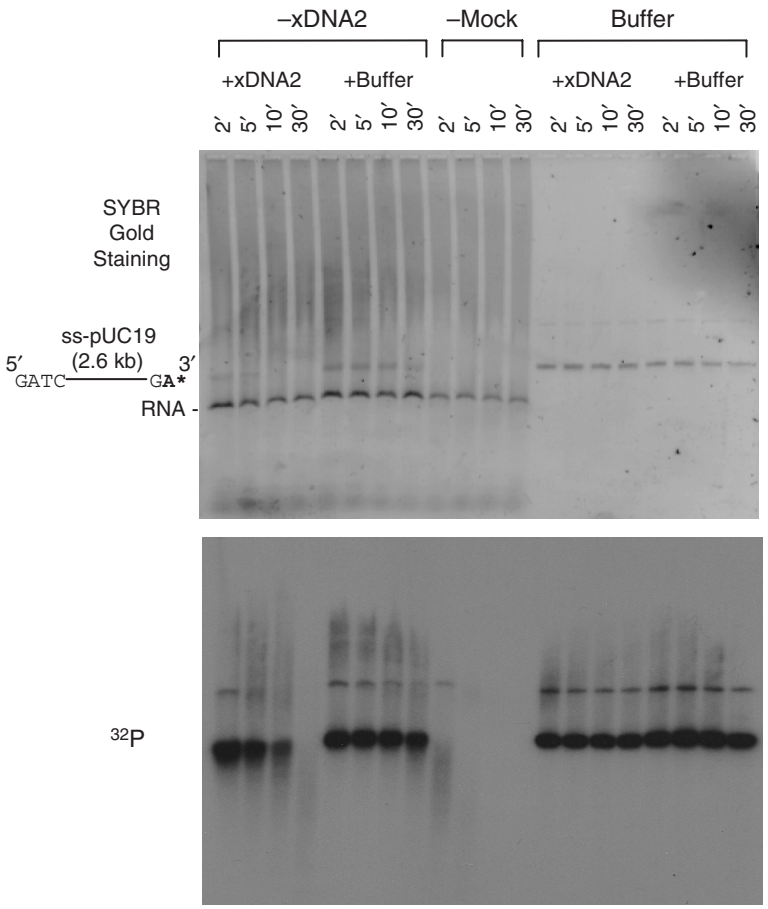

Figure 4. Effect of xDNA2 on 5′ ss-tail degradation. Denatured pUC19 DNA (2 ng/μl) labeled at the 3′-end was incubated in xDNA2-depleted or mock-depleted NPE supplemented with buffer or the purified xDNA2 (to ~5% of the level of the endogenous xDNA2). Time points were treated with SDS/proteinase K and separated on a 1% TAE agarose gel. The gel was first stained with SYBR Gold to detect total DNA and then dried for exposure to X-ray film to detect ^{32}P. The band migrated just below the ss-pUC19 DNA is the RNA in NPE.

Xenopus egg extracts. In a previous study, recombinant xDNA2 displayed only a very low level of ssDNA exonuclease activity (27). However, the difference between the two studies is superficial because, as shown in this study, the 5′→3′ ssDNA exonuclease activity is greatly stimulated by RPA, which was not used in the previous study. The role of xDNA2 in end processing was confirmed by the immunodepletion experiments showing that in the absence of xDNA2, end processing was significantly inhibited. In addition, depletion of xDNA2 caused a dramatic inhibition of SSA. Both defects could be complemented by the purified xDNA2 protein. Mechanistically, xDNA affects specifically the degradation of the 5′ ss-tail but not the unwinding of the DNA end. Functionally, the xDNA2-mediated end processing is not merely for degrading DNA but rather coupled to homology-dependent DSB repair. Taken together, these results indicate that, despite the lack of significant sequence homology between xDNA2 and RecJ, xDNA2 serves a function equivalent to what has been postulated for the *E. coli* RecJ exonuclease in end processing (Figure 7).

Yeast DNA2 has both helicase activity and ssDNA endonuclease activity, but only the nuclease activity is essential for viability (28,29). It appears to participate in Okazaki fragment maturation, telomere maintenance and DNA DSB repair (30). There is strong evidence that DNA2 and RecQ helicases interact functionally. A double mutant of DNA2 and SGS1, the yeast RecQ-type helicase, is conditionally lethal (31). Human WRN and BLM are both able to complement the yeast DNA2 mutant (32,33). There is also evidence that DNA2 might be involved in end processing. A double mutant of DNA2 and EXO1, which by itself has a certain albeit minor effect on end processing, is lethal (34). In addition, in the fission yeast *S. pombe*, DNA2 affects the 5′→3′ resection of telomeres, which are essentially natural DSBs (35). Interestingly, the intermediates in end processing and Okazaki fragment maturation are both branched molecules and in both cases it is the 5′ ss-branch that is cleaved. Previous studies have suggested that DNA2 is the nuclease that cleaves long 5′ branches sometimes generated during Okazaki fragment maturation (36). Notably, in this capacity, DNA2 does not act as a classical flap endonuclease like FEN1 to cleave at the branch point but rather as an exonuclease to shorten the 5′ branch. This role for DNA2 is essentially the same as that in end processing. In light of these observations, we conclude that our finding that xDNA2 is important for DNA end processing in the

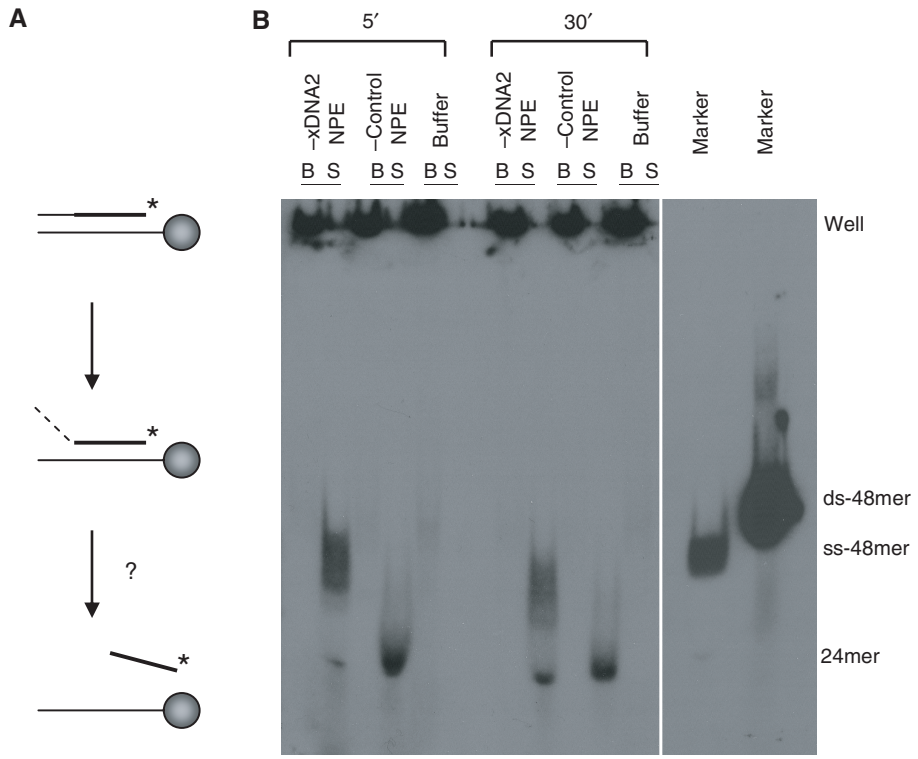

Figure 5. Effect of xDNA2 on end unwinding. (**A**) Principle of the unwinding assay. Thin line: normal nucleotides; thick line: thio nucleotides; *, ^{32}P label. (**B**) The thio 5′ oligo duplex precoated onto Streptavidin magnetic beads was incubated in xDNA2-depleted or mock-depleted NPE. Samples were separated into bead and supernatant fractions, treated with SDS/proteinase K and analyzed on a 10% native TAE PAGE. B, beads; S, supernatant. The SDS/proteinase K treatment in this study was at room temperature for 2 h, a condition not harsh enough to disrupt the binding of biotin with Streptavidin (1).

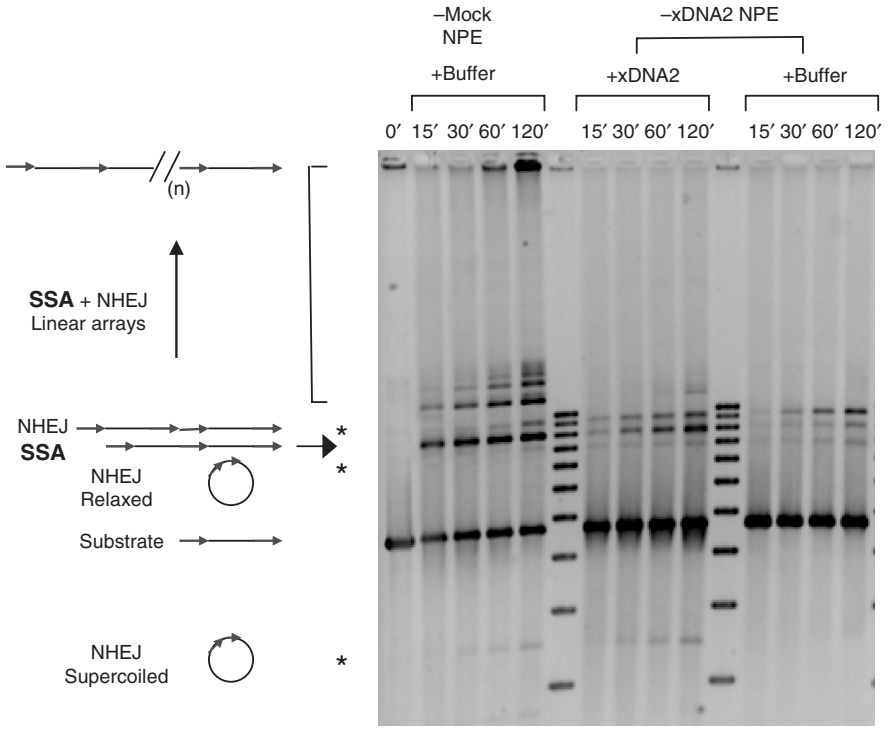

Figure 6. Effect of xDNA2 on SSA. The substrate, pRW4′, which carried two direct repeats at the partially filled-in XhoI protruding ends, was incubated in mock-depleted or xDNA2-depleted NPE (supplemented with xDNA2 or buffer) at room temperature. DNA samples were taken at the indicated times, treated with SDS/EDTA/Proteinase K, separated by TAE/agarose gel electrophoresis and detected by SYBR Gold stain. The SSA products include the band indicated by the arrow and a subset of the bands indicated by the bracket. The asterisks indicate the NHEJ products.

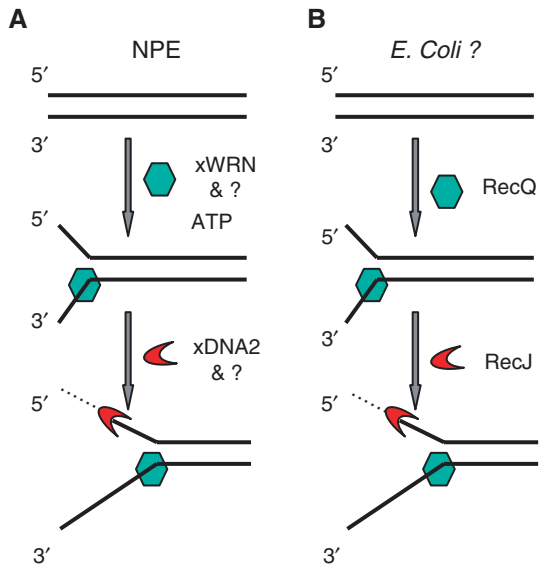

Figure 7. Model for the strand-specific processing of DNA ends. (**A**) End processing in the nucleoplasmic extract. xWRN (and potentially other helicases) are recruited to unwind the end in an ATP-dependent reaction. xDNA2 (and another exonuclease(s)) degrades the 5′ ss-tail. Proteins like MRE11 and CtIP (not depicted) might act upstream of xWRN. (**B**) The postulated RecQ-RecJ mechanism for end processing.

Xenopus system is consistent with genetic studies in yeast and the known mechanistic role of DNA2.

In summary, our biochemical studies in the *Xenopus* system have suggested that xWRN and xDNA2 work in sequence to execute the strand-specific end processing by acting respectively in end unwinding and 5′ ss-tail degradation. While these two proteins appear to constitute the basic mechanical components, they cannot be the only proteins for end processing. Depletion of neither xWNR nor xDNA2 led to a complete block to end processing, suggesting that there might exist other helicases and nucleases that could perform similar functions. In addition, WRN requires a 3′ ss-tail to initiate unwinding, but natural DSBs are expected to be blunt ended or nearly blunt ended. This suggests that there might be other proteins to load WRN to DSBs. One tantalizing possibility is that the MRN complex, which can rapidly bind to DSB ends, and Sae2/CtIP, which interacts with MRN, might serve such a role in the early stage of end processing (15,16,37). Potentially, MRN, in concert with other proteins like Sae2/CtIP, might recruit WRN to DNA ends by direct protein–protein interaction or by partially unwinding ends to provide the landing pad for WRN (9,38). The *Xenopus* system should provide an excellent opportunity for the elucidation of the mechanistic roles of these proteins and for the identification of additional proteins for end processing.

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