Supplemental Material

BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA-break repair

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Itemized list showing relation between supplemental and main figures.

Figure S1, related to Figure 1

Figure S2, related to Figure 1

Figure S3, related to Figure 2

Figure S4, related to Figure 3 and 4

Figure S5, related to Figure 4

Figure S6, related to Figure 4

Supplemental Materials and Methods

Enzymes and Reagents

*Cla*I, *Eco*RI, T4 polynucleotide kinase and Klenow fragment (3'→5' exo$^-$) were purchased from New England Biolabs. Terminal deoxynucleotidyl transferase and *Pfu Turbo* polymerase were purchased from Amersham and Stratagene respectively. ATP was purchased from Sigma and dissolved as a concentrated stock at pH 7. The concentration of the stock solution was determined using extinction coefficient of 15,400 $M^{-1}cm^{-1}$. [α-^{32}P]-dATP (3000 Ci/mmol) and [γ-^{32}P]-ATP (6000 Ci/mmol) were purchased from NEN Biochemicals. Proteinase K was purchased from Roche.

DNA and Proteins

Plasmid DNA (pUC19) was purified using a protocol that used lysis at a neutral pH followed by cesium chloride density gradient centrifugation (Sambrook et al. 1989). Its concentration was determined using an extinction coefficient of 6.6 x 10^3 $M^{-1}cm^{-1}$. Oligodeoxyribonucleotides X12-3 (GACGTCATAGACGATTACATTGCTAGGACATGCTGTCTAGAGACTATCGC), X12-3SC (TTGCTAGGACATGCTGTCTAGAGACTATCGC), X12-4C (GCGATAGTCTCTAGACAGCATGTCCTAGCAATGTAATCGTCTATGACGTC), X124NC (GCGATAGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGGCTA), AA03 (ACTC*GAATTC*CTCGAGGTCCCTTTATGGTCCC) and AA04 (CTGT*GAATTC*AAGCTTATCGATACCGTCACACCA) were synthesized by Sigma and purified by 20% polyacrylamide gel electrophoresis. Their concentrations were determined using extinction coefficients of 4.84 x 10^3, 2.95 x 10^3, 4.83 x 10^3, 4.88 x 10^3, 2.89 x 10^3 and 3.26 x 10^3 $M^{-1}cm^{-1}$ respectively. The oligonucleotides were ^{32}P-labeled at the 5'-ends (using [γ-^{32}P]-ATP and T4 polynucleotide kinase) or 3'-ends (using [α-^{32}P]-dATP and terminal deoxynucleotidyl transferase) according to vendor's protocol. The MRN complex (van der Linden et al. 2009), BLM (Nimonkar et al. 2008), Sgs1 (Cejka and Kowalczykowski 2010), *E. coli* RecQ (Harmon and Kowalczykowski 1998), DNA2 (wild type, K671E and D294A mutants) (Kim et al. 2006; Masuda-Sasa et al. 2006), Dna2 (Cejka et al. 2010), *E. coli* RecJ (Handa et al. 2009), EXO1 (wild type and D173A nuclease mutant) (Genschel et al. 2002; Dzantiev et al. 2004), human RPA (Walther et al. 1999), *S. cerevisiae* RPA (Kantake et al. 2003), and *E. coli* SSB (Harmon and Kowalczykowski 1998) were purified according to published protocols. To ensure that the

BLM and RPA preparations were free of contaminating nucleases, the proteins were incubated with ^{32}P-labeled 50-mer ssDNA and 50-bp dsDNA at molar concentrations that were ~50 fold higher than the DNA concentration (molecules); in the standard buffer and for standard incubation times, no nucleolytic degradation of the DNA was detected using denaturing gel electrophoresis. Human RecQ4, RecQ5, WRN, and BLM helicase mutant (K695R) were gifts from Patrick Sung (Yale University), Ian Hickson (University of Oxford, UK), Davis Chen (UT Southwestern) and Alex Mazin (Drexel University), respectively.

Substrates for nuclease assays

A 5'-end ^{32}P-labeled blunt-ended substrate (50 nucleotides (nt) long) was generated by annealing oligodeoxyribonucleotides X12-3 (^{32}P-labeled at 5'-end) and X12-4C which are completely complementary. A 3'-end ^{32}P-labeled blunt-ended substrate was generated similarly by annealing X12-3 and X12-4C (^{32}P-labeled at 3'-end). A 5'-end ^{32}P-labeled forked substrate (50 nt long, with 19 bases unpaired and 31 paired bases) was generated by annealing X12-3 (^{32}P-labeled at 5'-end) and X12-4NC, which are partially complementary. A 3'-end ^{32}P-labeled forked substrate was generated similarly by annealing X12-3 and X12-4NC (^{32}P-labeled at 3'-end). A 5'-end ^{32}P-labeled 3'-tailed substrate (50 nt long, 19 bases overhang and 31 paired bases) was generated by annealing X12-3SC (^{32}P-labeled at 5'-end) and X12-4C. Similarly, a 3'-end ^{32}P-labeled 3'-tailed substrate was generated by annealing X12-3SC and X12-4C (^{32}P labeled at 3' end). Annealing was performed as described previously (Cejka and Kowalczykowski 2010). *Eco*RI linearized pUC19 (2.7 kbp) was ^{32}P-labeled at the 5'-ends (using [γ-^{32}P]-ATP and T4 polynucleotide kinase) or 3'-ends (using [α-^{32}P]-dATP and Klenow fragment) according to the vendor's protocol. A 324 bp DNA fragment derived from the yeast *RAD51* gene promoter was generated

by PCR using *Pfu Turbo* polymerase as previously described (Alexeev et al. 2003) with the exception that the primers (AA03 and AA04) had *Eco*RI sites (indicated in italics). The amplification product was purified using Qiagen PCR cleanup kit. To label the fragment at the ends, the amplified product was first digested with *Eco*RI. *Eco*RI digested PCR fragment was ^{32}P-labeled at the 5'-ends (using [γ-^{32}P]-ATP and T4 polynucleotide kinase) or 3'-ends (using [α-^{32}P]-dATP and Klenow fragment) according to the vendor's protocol. After every radiolabeling reaction, the unincorporated nucleotides were separated from the labeled product using a G-25 spin column (GE). The concentration of the labeled product was determined by assuming ~95% recovery from G-25 spin column. DNA concentrations of the substrates are expressed in molar ends as well as nt. Schematic representations of the substrates are drawn on top of each figure. Red asterisk indicate position of ^{32}P on DNA ends.

EXO1 processivity assays

Reactions were performed at 37 °C and contained 1.2 nM gapped circular dsDNA (6.44 kb, gap size 314 nt (f1MR1 coordinates 6127-6440) and 5 mM $MgCl_2$. Reactions were done in the absence of ATP. Where indicated, BLM concentration was 20 nM. Reactions were started by addition of EXO1 to a concentration of 1.1 nM. To test for excision complex formation, f1MR1 ssDNA was added to a concentration of 6 nM molecules (38 μM nt) either 1 min after start of the reaction or at the start of the reaction. Reactions were stopped by the addition of 2 μg/μl Proteinase K and 1% SDS (final concentrations) and incubated for 30 min. DNA was recovered by ethanol precipitation after phenol extraction, digested with *Cla*I, electrophoresed under alkaline conditions, and transferred to nylon membrane (Hybond XL, GE Healthcare) as described (Genschel and Modrich 2003). Recession of the 5' end was detected by probing the

membranes with ^{32}P-labeled oligonucleotides hybridizing near the *Cla*I site (coordinates 2532-2552).The assay to determine the effect of MRN (20 nM) on the processivity of EXO1 (1 nM) was performed using a similar strategy with the exception that the standard nuclease assay with 2.7 kbp 3'-end ^{32}P-labeled DNA was used for analysis, and the competitor DNA was ϕX ssDNA (6 nM molecules, 32 μM nt).

Supplemental References

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Supplemental Figure Legends

Figure S1. BLM helicase requires RPA for unwinding activity, is blocked at higher concentrations of Mg^{2+} and the polarity of resection by BLM and DNA2 is 5'→3'.

Helicase reactions with BLM were performed using 3'-end labeled 2.7 kbp DNA.

(A) Requirement for RPA: All reactions contained 2 mM $MgCl_2$. Lanes 1-5: 0, 200, 400 and 800 nM RPA; substrate; Δ, heat-denatured substrate.

(B) The percentage of dsDNA unwound (obtained by expressing the amount of DNA unwound as a percentage of total signal) from experiments as shown in (A) plotted as a function of RPA concentration.

(C) Mg^{2+} concentration dependence: All reactions contained 400 nM RPA. Lanes: Δ, heat-denatured substrate; 1-5: 0, 1, 2, 5 and 10 mM $MgCl_2$.

(D) The percentage of dsDNA unwound (obtained by expressing the amount of DNA unwound as a percentage of total signal) from experiments as shown in (C) plotted as a function of Mg^{2+} concentration.

The positions of intact (2.7 kbp) and unwound (ssDNA) substrate (A and C) are indicated. Error bars (B and D) indicate standard deviation between 3 to5 independent experiments and are smaller than the symbols when not evident.

(E) and (F) Kinetics of resection.

(E) Nuclease reactions were performed using 3'-end labeled 2.7 kbp DNA. Lanes: 1-4, BLM; 5-8, BLM and DNA2; 9, DNA2; Δ, heat-denatured substrate. The positions of intact (2.7 kbp) and unwound (ssDNA) substrate, resection products, and molecular weight markers are indicated.

(F) Nuclease reactions were performed using 5'- or 3'-end labeled 324-bp DNA. All reactions contained 50 nM RPA and 2 mM $MgCl_2$. Lanes: 1-5, 5'-end labeled DNA; 6-10, 3'-end labeled

DNA. The positions of intact substrate (324 bp), resection products and contaminants in the PCR product (asterisk) are indicated.

Figure S2. The polarity of cleavage by DNA2 is 5' → 3'.

(A) Nuclease reactions were performed as described in Figure 1B with the exception that products were resolved on a 12% urea-polyacrylamide gel. All reactions contained 10 nM RPA and 5 mM $MgCl_2$. Lanes: 1, forked substrate; 2, forked substrate (5'-end labeled) plus DNA2; 3, forked substrate (3'-end labeled) plus DNA2. Lanes 2 and 3 are the same as lanes 4 and 7 in Figure 1B. The positions of the intact 50-mer, cleavage product and markers (dT_{50}, dT_{25} and dT_{12}) are indicated.

(B) RPA promotes 5'→3' degradation and inhibits 3'→5' degradation of a flapped DNA substrate by DNA2.

Nuclease assays with DNA2 were performed using duplex DNA substrates containing 5'- or 3'-ssDNA flap. RPA was added to the indicated concentration. The reactions were incubated for 1 hr at 37 ^{o}C. The products were denatured by heat and resolved using 12% SequaGel and detected by autoradiography. The positions of markers are indicated.

Figure S3. Resection mediated by BLM and DNA2 is endonucleolytic, requires RPA, and a cognate helicase.

Nuclease reactions were performed using 5'-end labeled 50 bp DNA fragment.

(A) BLM-DNA2 mediated resection in not exonucleolytic.

(B) Requirement for RPA during resection.

(C) and (D) RecQ4 (Q4), WRN (W) and RecQ5 (Q5) helicase do not substitute for BLM (B). The intrinsic nuclease activity of WRN was weakly evident (lanes 5 and 6, panel D).

(E) Sgs1 helicase is inhibited by higher Mg^{2+} concentration. The percent of DNA unwound is

plotted as a function of Mg^{2+} concentration. The amount of substrate unwound was expressed as a percentage of total signal. Error bars indicate standard deviation from 3 independent experiments.

Figure S4. MRN stimulates DNA unwinding by BLM and resection by EXO1.

(A) Stimulation of BLM helicase by MRN. Lanes: 1, substrate; 2, MRN; 3, BLM; 4 BLM and MRN; Δ, heat-denatured substrate. The positions of the intact (2.7 kbp) and unwound (ssDNA) substrate are indicated.

(B) The percent of unwound substrate from experiments as shown in Figure 3A plotted as a function of MRN concentration. The amount of substrate unwound was expressed as a percentage of total signal. Error bars indicate standard deviation between 3 to 5 independent experiments.

(C) MRN stimulates BLM-mediated unwinding of blunted ended DNA. Helicase reactions with BLM (0, 2.5, 5 and 10 nM) were performed using 3'-end labeled blunt-ended DNA. All reactions contained 10 nM RPA and 5 mM $MgCl_2$. Lanes: 1-4, BLM alone; 5-8, BLM and MRN; 9, substrate; Δ, heat-denatured substrate. The positions of the intact (50 bp) and unwound (50 nt) substrate are indicated.

(D) MRN stimulates BLM-mediated unwinding of 3'-tailed DNA. Helicase reactions were performed as described in (C) using 3'-end labeled tailed DNA (1.5 nM ends, 60 nM nt). Lanes: 1-4, BLM alone; 5-8, BLM and MRN; 9, substrate; Δ, heat-denatured substrate.

(E) MRN and EXO1 resect DNA in the absence of ATP. All reactions contained 5 mM $MgCl_2$. RPA was included when indicated. Lanes: 1-3, EXO1, 4-6, MRN and EXO1, 7-9, MRN, EXO1 and RPA; 10-12, EXO1 and RPA. The positions of the intact substrate (2.7 kbp) and resection products are indicated.

(F) Stimulation of EXO1 by MRN is not species-specific. Where indicated, MRN was substituted with yeast MRX. All reactions contained 5 mM $MgCl_2$ and 200 nM RPA. Lanes: 1-3, EXO1, 4-6, EXO1 and MRN; 7-9 and 10-12, EXO1 and MRX; 13-15, MRX. The positions of the intact substrate (2.7 kbp) and resection products are indicated.

Figure S5. The use of Mn^{2+} to activate MRN nuclease blocks resection.

Nuclease reactions with MRN, EXO1, BLM, DNA2 and RPA were performed using 3'-end labeled 2.7 kbp DNA. The concentrations of MRN, EXO1, BLM, DNA2 and RPA were 10 nM, 2.5 nM, 10 nM, 4 nM and 200 nM respectively. The concentration of $MgCl_2$ or $MnCl_2$ is indicated. (A) Substitution of Mn^{2+} for Mg^{2+} inhibits resection: Lanes: 1-6, reactions in Mg^{2+}; 7-15, reactions in Mn^{2+}. (B) Addition of Mn^{2+} inhibits DNA end resection: Lanes: 1-9, reactions in a fixed concentration of Mg^{2+} and various concentrations of Mn^{2+}. The positions of intact substrate (2.7 kbp) and resection products are indicated.

Figure S6. BLM increases the affinity of EXO1 for DNA ends but not the processivity.

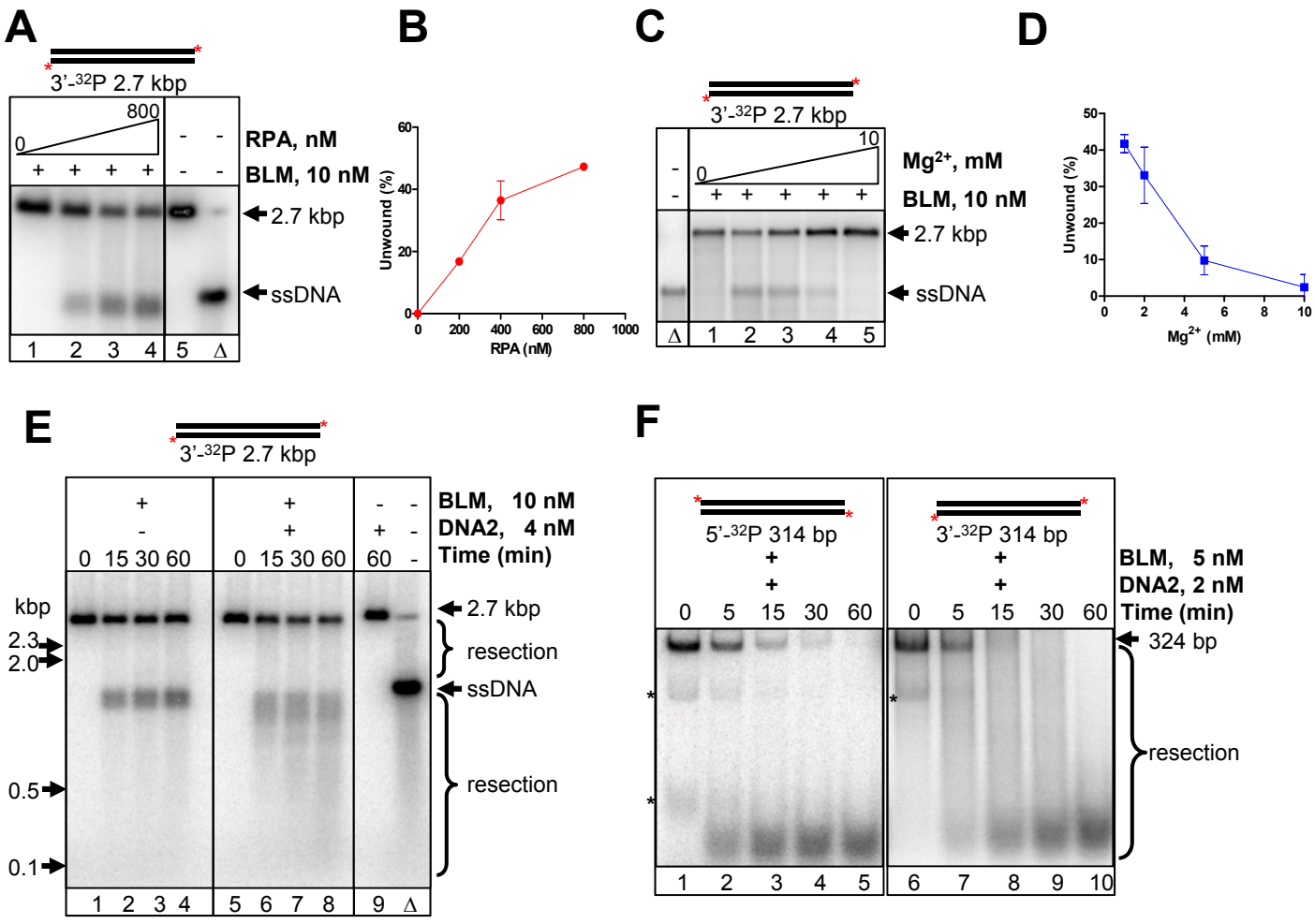
Processivity was measured as described in Materials and Methods.

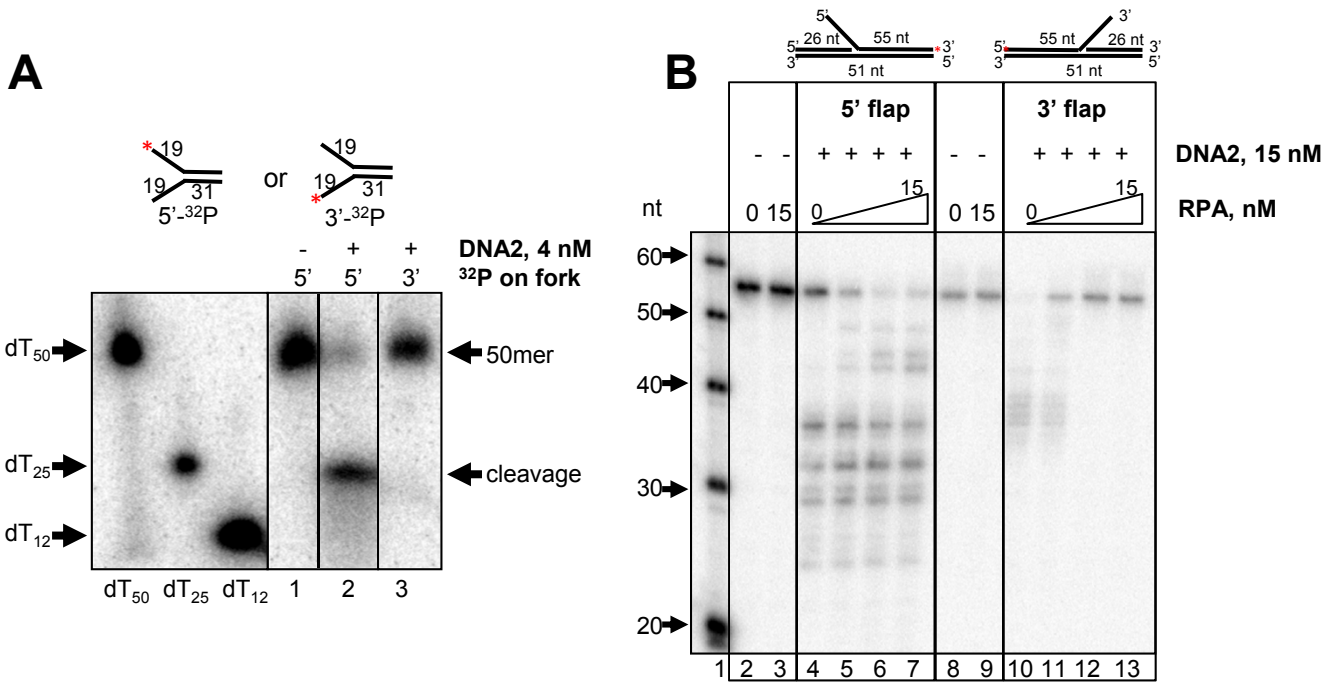
(A) Kinetics of EXO1 activity. Lanes: 1-5, no ssDNA added; 6-10, ssDNA added at 0 min; 11-15, ssDNA added after 1 min pre-incubation.

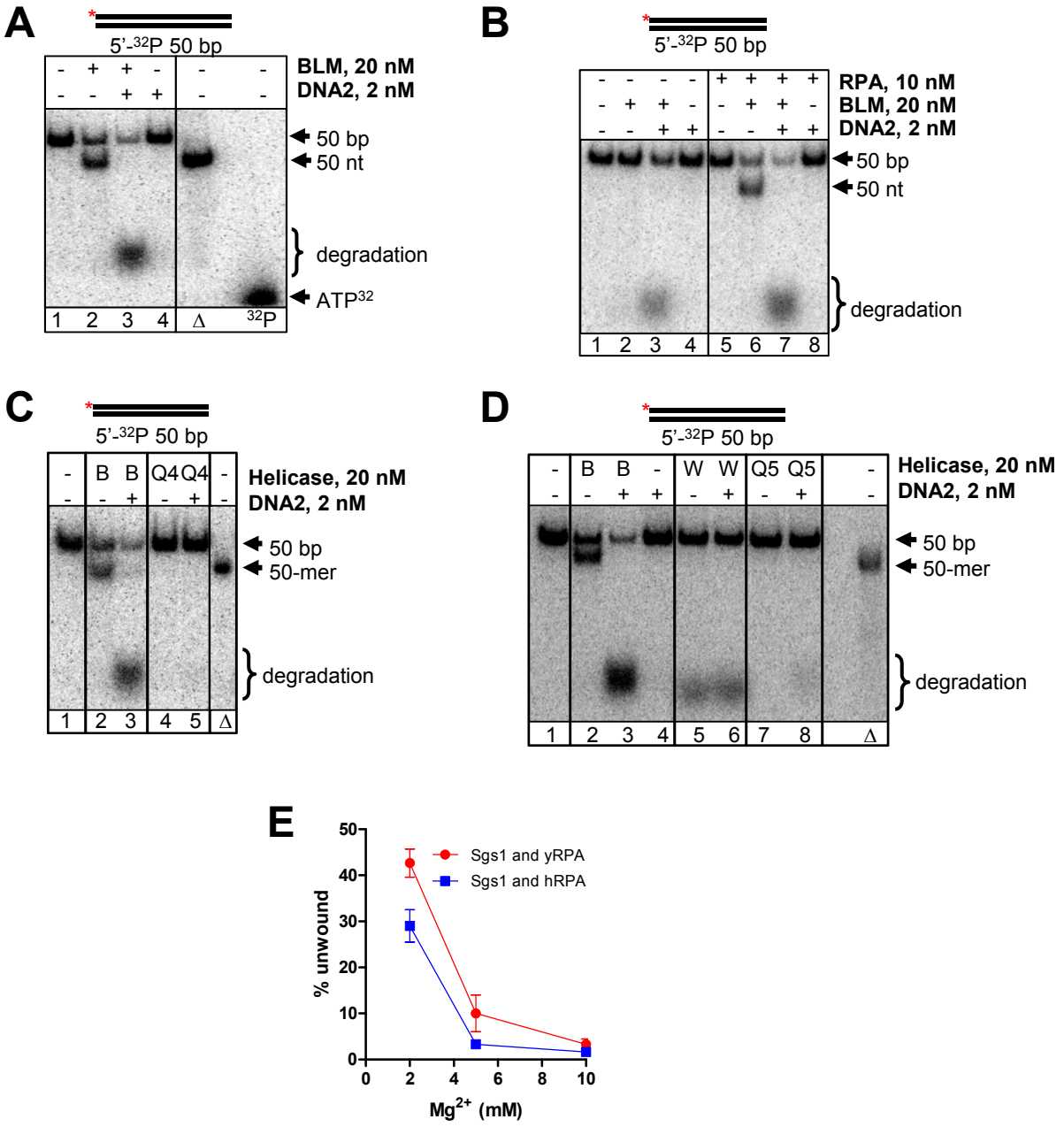
(B) BLM has no nuclease activity. Lanes: 1-5, time course with BLM alone.

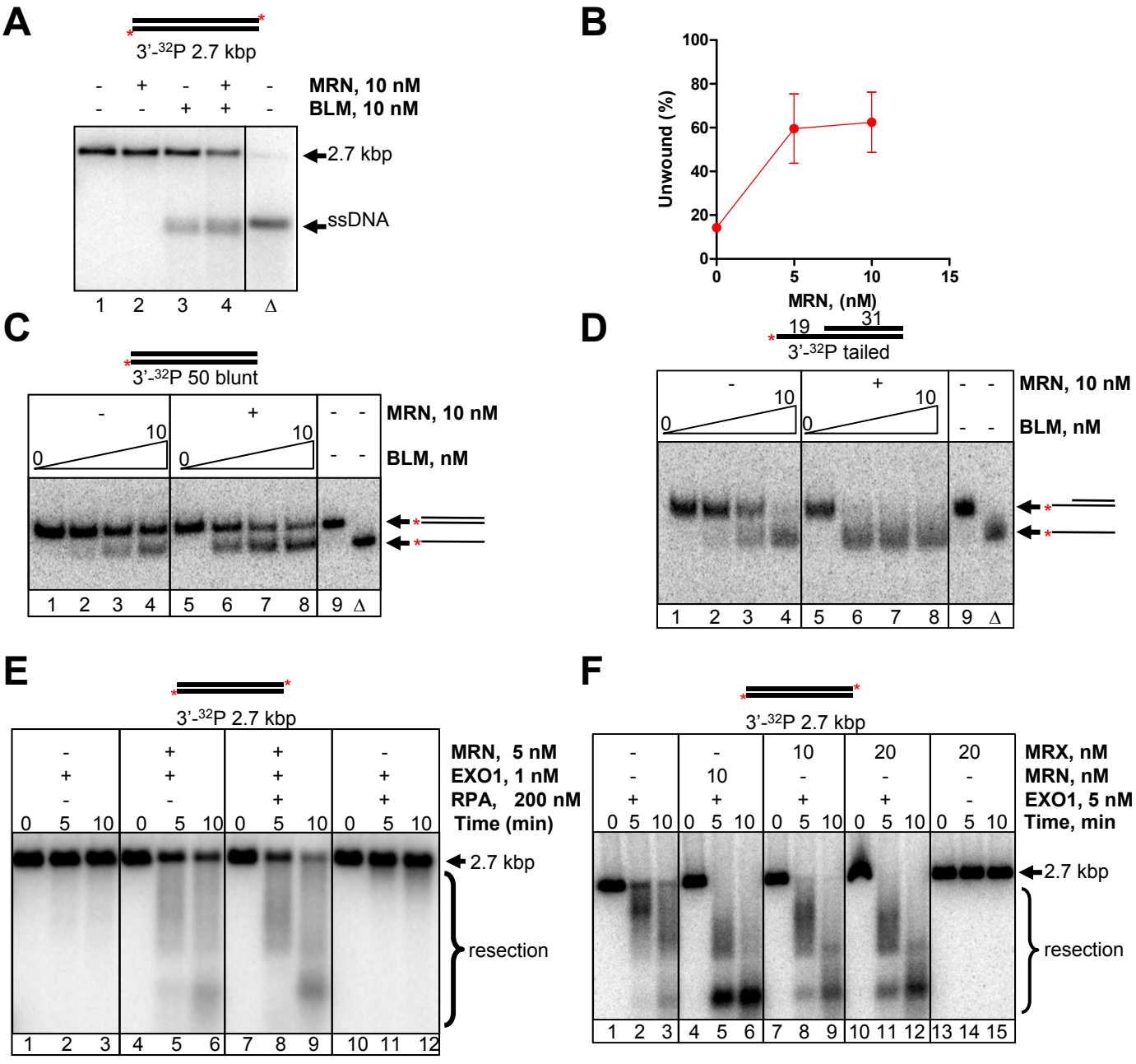
(C) Kinetics of EXO1 activity in the presence of BLM. Lanes: 1-5, no ssDNA added; 6-10, ssDNA added at 0 min; 11-15, ssDNA added after 1 min pre-incubation.

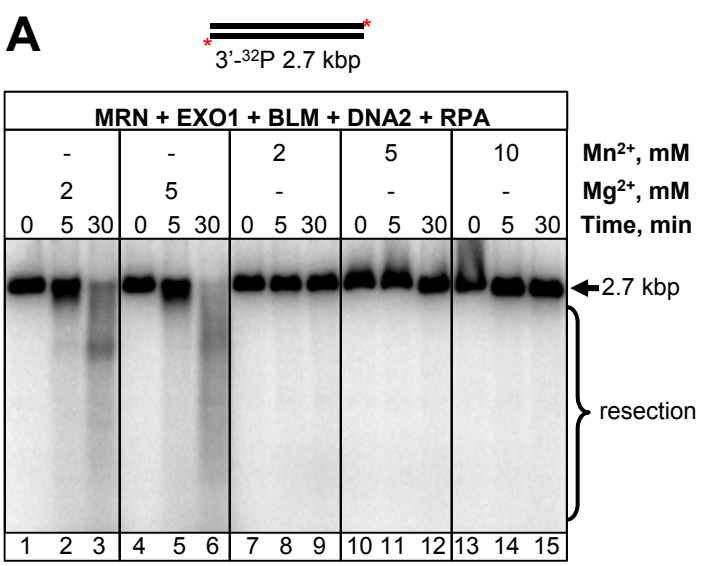
The positions of DNA fragment corresponding to no degradation (3600 nt), the resection intermediates and markers (nt) are indicated.

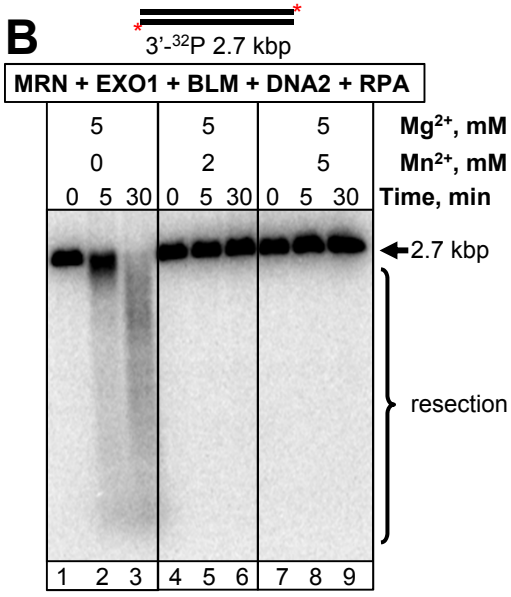

A
3'-32P 2.7 kbp
800
0
RPA, nM
BLM, 10 nM
2.7 kbp
ssDNA
1 2 3 4 5 Δ
B
Unwound (%)
RPA (nM)
C
3'-32P 2.7 kbp
10
0
Mg2+, mM
BLM, 10 nM
2.7 kbp
ssDNA
Δ 1 2 3 4 5
D
Unwound (%)
Mg2+ (mM)
E
3'-32P 2.7 kbp
BLM, 10 nM
DNA2, 4 nM
Time (min)
0 15 30 60 0 15 30 60 60
kbp
2.3
2.0
0.5
0.1
2.7 kbp
resection
ssDNA
resection
1 2 3 4 5 6 7 8 9 Δ
F
5'-32P 314 bp
3'-32P 314 bp
BLM, 5 nM
DNA2, 2 nM
Time (min)
0 5 15 30 60 0 5 15 30 60
324 bp
resection
1 2 3 4 5 6 7 8 9 10

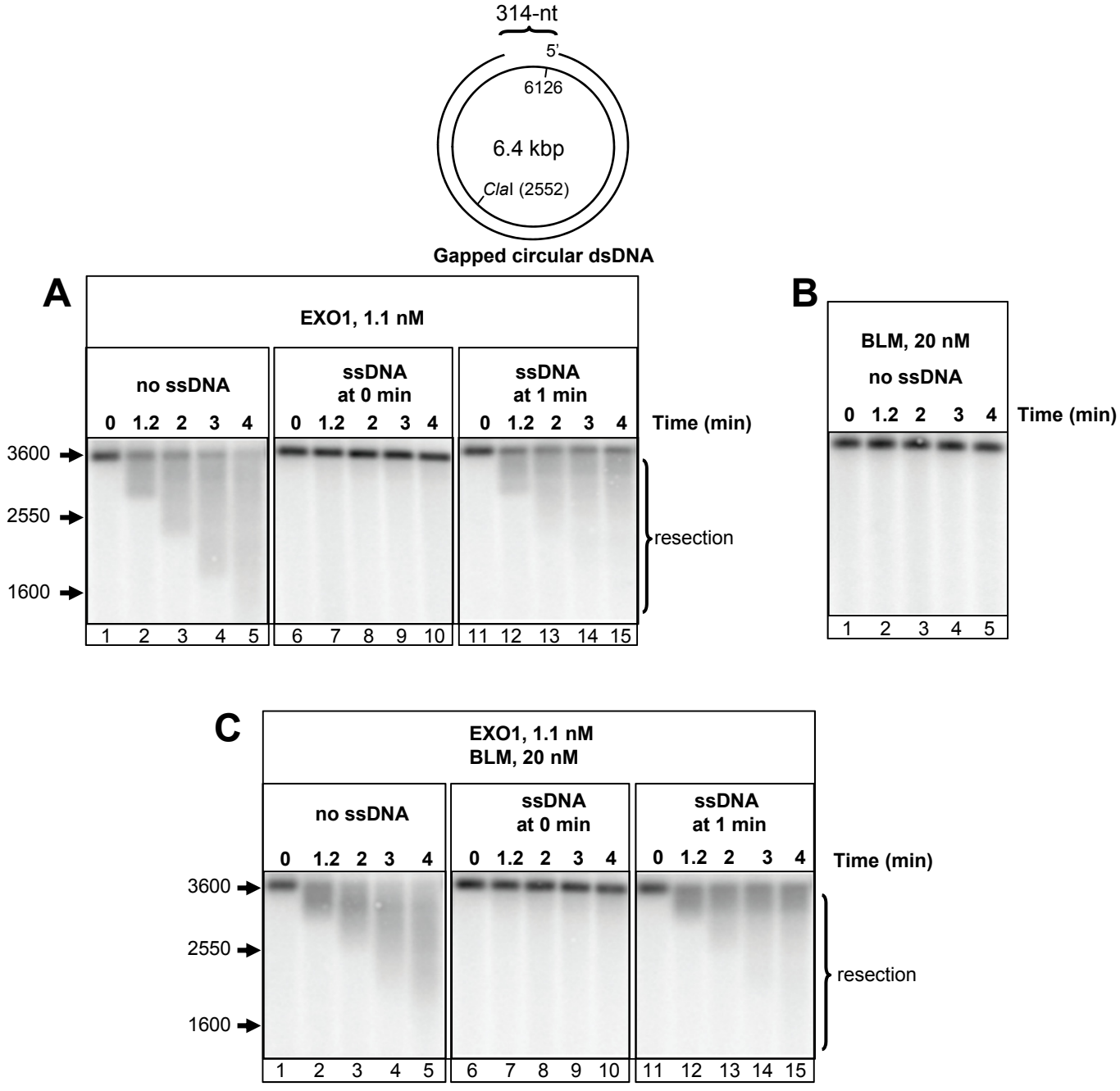

A
19
19
31
5'-32P
or
19
19
31
3'-32P
DNA2, 4 nM
32P on fork
-
+
+
5'
5'
3'
dT50
dT25
dT12
50mer
cleavage
dT50 dT25 dT12 1 2 3
B
5'
26 nt
55 nt
3'
5'
3'
51 nt
5'
55 nt
26 nt
3'
5'
3'
51 nt
5' flap
3' flap
DNA2, 15 nM
RPA, nM
nt
0 15
0
15
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10 11 12 13


A
5'-32P 50 bp
BLM, 20 nM
DNA2, 2 nM
50 bp
50 nt
degradation
ATP32
1 2 3 4 Δ 32P
B
5'-32P 50 bp
RPA, 10 nM
BLM, 20 nM
DNA2, 2 nM
50 bp
50 nt
degradation
1 2 3 4 5 6 7 8
C
5'-32P 50 bp
Helicase, 20 nM
DNA2, 2 nM
50 bp
50-mer
degradation
1 2 3 4 5 Δ
D
5'-32P 50 bp
Helicase, 20 nM
DNA2, 2 nM
50 bp
50-mer
degradation
1 2 3 4 5 6 7 8 Δ
E
Sgs1 and yRPA
Sgs1 and hRPA
% unwound
Mg2+ (mM)
0 10 20 30 40 50
0 2 4 6 8 10


A
3'-32P 2.7 kbp
MRN, 10 nM
BLM, 10 nM
2.7 kbp
ssDNA
1 2 3 4 Δ
B
Unwound (%)
MRN, (nM)
C
3'-32P 50 blunt
MRN, 10 nM
BLM, nM
1 2 3 4 5 6 7 8 9 Δ
D
19
31
3'-32P tailed
MRN, 10 nM
BLM, nM
1 2 3 4 5 6 7 8 9 Δ
E
3'-32P 2.7 kbp
MRN, 5 nM
EXO1, 1 nM
RPA, 200 nM
Time (min)
2.7 kbp
resection
1 2 3 4 5 6 7 8 9 10 11 12
F
3'-32P 2.7 kbp
MRX, nM
MRN, nM
EXO1, 5 nM
Time, min
2.7 kbp
resection
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


A
3'-32P 2.7 kbp
MRN + EXO1 + BLM + DNA2 + RPA
- - 2 5 10
Mn2+, mM
2 5 - - -
Mg2+, mM
0 5 30 0 5 30 0 5 30 0 5 30 0 5 30
Time, min
2.7 kbp
resection
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
3'-32P 2.7 kbp
MRN + EXO1 + BLM + DNA2 + RPA
5 5 5
Mg2+, mM
0 2 5
Mn2+, mM
0 5 30 0 5 30 0 5 30
Time, min
2.7 kbp
resection
1 2 3 4 5 6 7 8 9


314-nt
5'
6126
6.4 kbp
ClaI (2552)
Gapped circular dsDNA
A
EXO1, 1.1 nM
no ssDNA
ssDNA at 0 min
ssDNA at 1 min
0 1.2 2 3 4
Time (min)
3600
2550
1600
resection
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
BLM, 20 nM
no ssDNA
0 1.2 2 3 4
Time (min)
1 2 3 4 5
C
EXO1, 1.1 nM
BLM, 20 nM
no ssDNA
ssDNA at 0 min
ssDNA at 1 min
0 1.2 2 3 4
Time (min)
3600
2550
1600
resection
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15