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# Repair of double-strand breaks by nonhomologous end joining in the absence of Mre11

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Mre11–Rad50–Nbs1 (MRN) complex involvement in nonhomologous end joining (NHEJ) is controversial. The MRN complex is required for NHEJ in *Saccharomyces cerevisiae* but not in *Schizosaccharomyces pombe*. In vertebrates, *Mre11*, *Rad50*, and *Nbs1* are essential genes, and studies have been limited to cells carrying hypomorphic mutations in Mre11 or Nbs1, which still perform several MRN complex–associated activities. In this study, we analyze the effects of Mre11 loss on the mechanism of vertebrate NHEJ by using a chromatinized plasmid double-strand break (DSB) repair assay in cell-free extracts from *Xenopus laevis*. Mre11-depleted extracts are able to support efficient NHEJ repair of DSBs regardless of the end structure. Mre11 depletion does not alter the kinetics of end joining or the type and frequency of junctions found in repaired products. Finally, Ku70-independent end-joining events are not affected by Mre11 loss. Our data demonstrate that the MRN complex is not required for efficient and accurate NHEJ-mediated repair of DSBs in this vertebrate system.

## Introduction

Mammalian cells rely on the nonhomologous end-joining (NHEJ) pathway to repair most of their DNA double-strand breaks (DSBs), the most harmful type of DNA damage (Khanna and Jackson, 2001). NHEJ is a mechanism in which broken ends at a DSB site are rejoined without requiring extended segments of homology. The main components of the vertebrate NHEJ machinery comprise DNA ligase IV, XRCC4, Artemis, and the DNA-dependent protein kinase (DNA-PK), which is composed of a large catalytic subunit, DNA-PKcs, and the Ku70/80 heterodimer. Defects in NHEJ factors result in radiosensitivity and immunodeficiency syndromes, as vertebrate lymphocytes rely on the NHEJ pathway to complete the rejoining step of V(D)J recombination (Taccioli et al., 1993; Nussenzweig et al., 1996).

The MRN/MRX complex is a multisubunit nuclease that is composed of Mre11, Rad50, and Nbs1 (Xrs2 in *Saccharomyces cerevisiae*). Mutations in genes encoding these proteins result in genomic instability, increased sensitivity to ionizing radiation, telomere shortening, and meiosis defects (D'Amours and Jackson, 2002). In *S. cerevisiae*, HO-induced NHEJ-dependent cell survival is reduced in *mre11*, *rad50*, or *xrs2* mutants (Moore and Haber, 1996). Mutations in any of the MRN/X complex components display a 40–50-fold reduction in the efficiency of plasmid end joining in *S. cerevisiae* (Boulton and Jackson, 1998) but show no significant defects in *Schizosaccharomyces pombe* (Manolis et al., 2001).

*Mre11*, *Rad50*, and *Nbs1* are all essential genes in vertebrates (Xiao and Weaver, 1997; Luo et al., 1999; Zhu et al., 2001). In vitro studies with extracts from mammalian cells indicated a requirement for the MRN complex in the NHEJ pathway (Huang and Dynan, 2002; Zhong et al., 2002). In contrast, no DSB repair deficiency has been observed in human or mouse cells harboring hypomorphic mutations in the *Mre11* or *Nbs1* gene, which are responsible for Ataxia telangiectasia–like disorder (ATLD) or Nijmegen breakage syndrome, respectively (Varon et al., 1998; Stewart et al., 1999). However, a subset of MRN complex activities is still associated with ATLD and Nijmegen breakage syndrome alleles (Stewart et al., 1999; Theunissen et al., 2003; Costanzo et al., 2004). Therefore, these experiments could not rule out a role for the MRN complex in NHEJ.

*Xenopus laevis* egg extracts support highly efficient and precise end joining of linear DNA molecules using similar pathways to those found in mammalian cells (Pfeiffer and Vielmetter, 1988; Thode et al., 1990; Sandoval and Labhart, 2002). These extracts have been used extensively to characterize the role of the Ku protein in eukaryotic NHEJ (Labhart, 1999; Sandoval and Labhart, 2002). In this study, we show that in contrast to Ku70-depleted extracts, Mre11-depleted extracts are competent to end join with high efficiency regardless of the

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Abbreviations used in this paper: ATLD, Ataxia telangiectasia–like disorder; CC, closed circle; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; LD, linear dimer; MRN, Mre11–Rad50–Nbs1; NHEJ, nonhomologous end joining; PSS, protruding single strand.

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DNA end structure. We find that the kinetics of end joining as well as the sequence of junctions formed is comparable in the absence of Mre11 and in mock-depleted extracts. Together, our data indicate that the MRN complex plays no detectable role in the repair of DSBs by the NHEJ pathway.

## Results and discussion

### Immunodepletion of Mre11 and Ku70 proteins from *X. laevis* egg extracts

We investigated the consequences of Mre11 inactivation on NHEJ by depleting Mre11 protein from cell-free extracts with a polyclonal serum specific for *X. laevis* Mre11 protein (anti-XMre11). We previously used these antibodies to demonstrate that genomic DNA accumulates DSBs during DNA replication in Mre11-depleted extracts (Costanzo et al., 2001). Moreover, Mre11 depletion dramatically impairs the ATM (Axatia telangiectasia mutated)-dependent response to DSBs (Costanzo et al., 2004). Importantly, we show that the former defect is rescued by the Mre11 hypomorphic ATLD3/4 allele, whereas ATM activation is not (Costanzo et al., 2004). This clearly demonstrates that Mre11-associated activity remains in ATLD cells. We used two types of cell-free extracts for our study and established that egg cytosol and membrane-free egg cytosol supported NHEJ with similar efficiency (see Figs. 2 and 4).

Mre11 protein was quantitatively removed by two rounds of immunodepletion and was no longer detectable by Western blotting (Fig. 1 A, lanes 2–5; and B). Mre11 depletion did not affect Ku70 protein level (Fig. 1 A, lanes 2–5). Conversely, Mre11 protein levels were not reduced by Ku70 depletion (Fig. 1 A, lanes 6–9). This demonstrates that there is no significant interaction between the two proteins in solution, and it allows for the independent analysis of Mre11- and Ku70-depleted extracts. Moreover, both Mre11 and Ku70 proteins can be depleted simultaneously from egg extracts (Fig. 1 C).

All assays were performed after two rounds of immunodepletion. The extent of Mre11 depletion was assessed by incubating 20 μl of depleted extracts with linear biotinylated DNA molecules immobilized on streptavidin beads. The total fraction of DNA-bound proteins was then pulled down and analyzed by Western blotting. No Mre11 protein signal was detected in either Mre11- or Mre11- and Ku70-depleted extracts as compared with the untreated egg cytosol (Fig. 1 D).

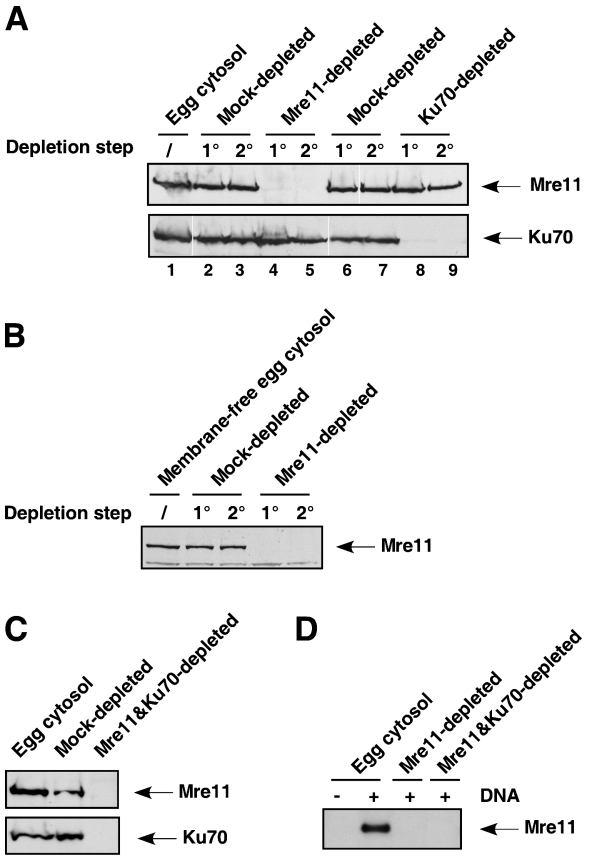


Figure 1. **Immunodepletion of Mre11 and Ku70 proteins from *X. laevis* egg extracts.** (A) Untreated egg cytosol and mock-, Mre11-, or Ku70-depleted extracts were analyzed by Western blotting with either anti-XMre11 serum or antibodies against Ku70. (B) Untreated membrane-free egg cytosol and extracts treated with preimmune (mock depleted) or anti-XMre11 serum (Mre11 depleted) were analyzed by Western blotting with anti-XMre11 serum. (C) Mre11 and Ku70 double-depleted egg cytosol (Mre11 and Ku70 depleted) were analyzed with either anti-XMre11 serum or antibodies against Ku70. (D) Untreated egg cytosol, Mre11-, or Mre11 and Ku70 double-depleted extracts were incubated with streptavidin beads coated with biotinylated, linear double-strand DNA, and the fraction of proteins bound to DNA was analyzed by Western blotting with anti-XMre11 serum.

### Mre11 is not required for efficient NHEJ

Both Mre11- and Ku70-depleted extracts were tested for NHEJ activity. Ku70 depletion provided a control for the removal of a protein required for NHEJ (Fig. 2, A and B). Nine different NHEJ substrates were generated by digesting the pBS KS II plasmid with one or two different restriction enzymes to yield linear DNA molecules (Table SI, available at http://www.jcb.org/cgi/content/full/jcb.200506029/DC1). The NHEJ substrates were individually incubated in extracts at a final concentration of 1 ng/μl, and samples were taken at 0 and 150 min. Upon incubation in control extracts (Fig. 2 A, untreated egg cytosol and mock-depleted extracts), the linear molecules underwent efficient intramolecular NHEJ, generating covalently closed circles (CCs). CCs were the prominent NHEJ product in control extracts (Fig. 2 A and Fig. S1). In addition to recircularization, intermolecular joining of the linear substrates generated minor products: linear dimer (LD) as well as higher multimeric forms (collectively indicated as M). Ku70 depletion dramatically impaired intramolecular end joining, and CC forms were not detected (Fig. 2 A, lanes 10, 11, 21, and 22). As previously described (Labhart, 1999), multimerization of linear substrates was favored in these extracts. This pattern of reduced circularization and increased multimer formation after Ku70 depletion was observed with all NHEJ substrates (unpublished data). Next, we assessed whether the catalytic activity of DNA-PK was required by using NU7026, a specific inhibitor of DNA-PKcs (Hollick et al., 2003). We found that intramolecular end joining was inhibited at doses as low as 0.5 μM, whereas intermolecular end joining was inhibited at 25 μM NU7026 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200506029/DC1). Our results suggest that DNA-PKcs is also required for NHEJ. In

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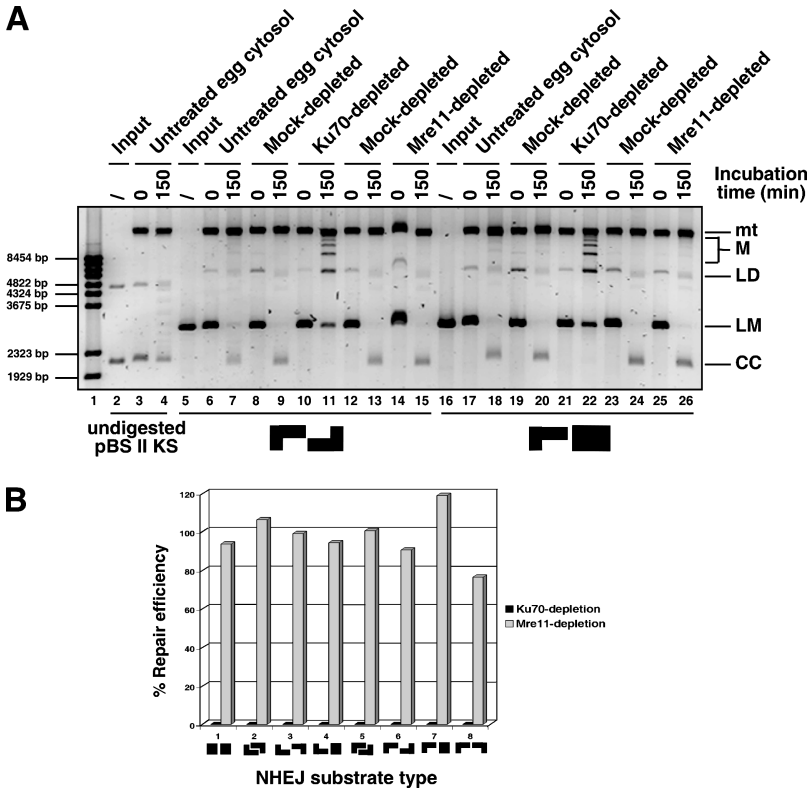


Figure 2. **Mre11 is not required for efficient NHEJ.** (A) Representative SYBR gold–stained gel characterization of NHEJ products for substrate types nonmatching 3′-PSS (lanes 5–15) and blunt end + 3′-PSS (lanes 16–26). Lanes 5 and 16 show 10 ng of input SacI-KpnI and SacI-SmaI substrates, respectively. The input in lane 2 corresponds to 10 ng of undigested pBS KS II. mt, mitochondrial DNA from *X. laevis* extract; M, higher multimer forms; LD, linear dimer; LM, linear monomer (substrate); CC, closed circle. (B) The intensity of the bands corresponding to NHEJ products were quantified using the FluorImager system for each NHEJ reaction in Ku70- and Mre11-depleted extracts and were expressed as percent repair efficiency (see Results). The labeling on the x axis refers to the NHEJ substrate type as indicated in Table SI (available at http://www.jcb.org/cgi/content/full/jcb.200506029/DC1).

contrast, the profile of NHEJ product formation was not affected by Mre11 depletion and was indistinguishable from that of both untreated and mock-depleted extracts. In the absence of Mre11 protein, the linear substrates were efficiently converted to NHEJ products (Fig. 2 A, lanes 14, 15, 25, and 26). This strongly suggests that Mre11 is dispensable for NHEJ repair of these substrates.

To uncover any possible DNA end structure–dependent role of Mre11 in NHEJ, we determined the repair efficiency for each NHEJ substrate in Ku70- or Mre11-depleted extracts. Repair efficiency was defined as the ratio between recircularized product (CC) and total DNA (NHEJ products and remaining substrate) and was expressed as the percentage of the corresponding efficiency in mock-depleted extracts (Fig. 2 B, percent repair efficiency values). We used circular product formation by intramolecular end joining as a relevant measure for NHEJ activity in the extract because CC formation is dependent on Ku70 and DNA-PKcs (Fig. S2; Labhart 1999). In the absence of Ku70, the efficiency of the repair reaction was reduced to zero for all NHEJ substrates (Fig. 2 B), including cohesive 5′-protruding single strand (PSS) digested with EcoRI or BamHI (unpublished data), which differs from a previous study (Labhart, 1999). In contrast, the mean repair efficiency in Mre11-depleted extracts was 95.1% of the corresponding value in mock-depleted extracts, thus indicating that all reactions are highly efficient in the absence of Mre11 (Fig. 2 B). Similar results were obtained when NHEJ reactions were characterized by Southern blot hybridization (Fig. S1). Altogether, our data establish that Mre11 is not required for efficient NHEJ in *X. laevis* cell-free extract regardless of the type of substrate.

Next, we determined whether the formation of intermolecular products (dimers and multimers) that were preferentially observed after Ku70 depletion required Mre11. To that end, we depleted Ku70 and Mre11 simultaneously (Fig. 1 C). The formation of intermolecular NHEJ products (LD and M) in Ku70-depleted extracts (Fig. 3 A, lane 8) was not inhibited after further Mre11 depletion (Fig. 3 A, lane 12). Together, these data show that Mre11 is not required for the formation of intramolecular (CC) or intermolecular products (LD and M).

Next, we altered the molar ratio of proteins and substrate DNA and monitored the consequences on end joining. We first compared the consequences of Mre11 removal at different concentrations of NHEJ substrate. We found no significant difference between the controls and Mre11-depleted extracts when extracts were incubated with 0.1, 1, or 10 ng/μl NHEJ substrate (Fig. 3 A). At the higher concentration of DNA, we found that a NHEJ factor was limiting because only a fraction of template was rejoined. Moreover, the distribution of end-joining products was not altered when NHEJ was monitored in extracts supplemented with the recombinant MRN complex at a final concentration up to threefold the level of endogenous MRN complex (Fig. 3 B).

Recent in vitro studies in HeLa and *S. cerevisiae* reported a direct stimulatory interaction between the MRN complex and NHEJ core components (Chen et al., 2001; Huang and Dynan, 2002). Moreover, studies in fractionated mammalian cell extracts showed that extracts in which Rad50 activity was inhibited were defective in intermolecular end joining (Zhong et al., 2002). Unlike *X. laevis* extracts, these mammalian cell-free extracts do not support intramolecular end joining, as seen by the exclusive formation of dimers and higher multimers resulting

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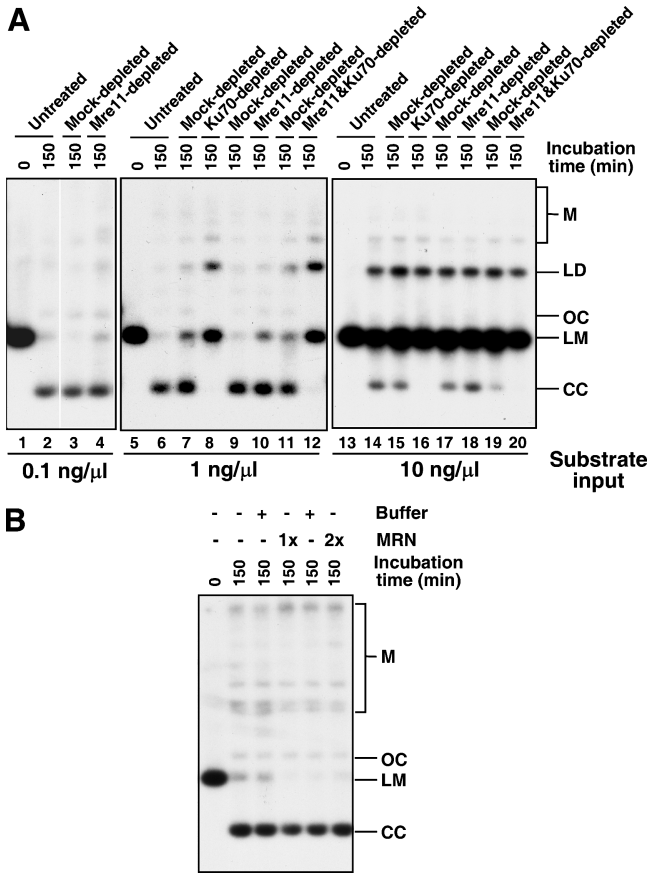


Figure 3. **Mre11 depletion does not affect NHEJ-mediated generation of multimers.** (A) Southern blot analysis of NHEJ products generated by incubation of blunt end + 5′-PSS substrate in the indicated depleted egg cytosol extracts at a substrate input concentration of 0.1 (left), 1 (middle), or 10 (right) ng/μl. Data in the left panel are derived from two segments of the same gel (as indicated by the dividing line). (B) Blunt end + 5′-PSS substrate was incubated at 1 ng/μl in untreated egg cytosol that had been supplemented with the recombinant MRN complex, and NHEJ products were analyzed by Southern blot hybridization. M, higher multimer forms; LD, linear dimer; LM, linear monomer (substrate); CC, closed circle; OC, open circle.

from intermolecular ligation. Furthermore, cell-free extracts from *X. laevis* eggs differ from an in vitro system or a fractionated cell extract in that they contain all cytosolic and nuclear proteins normally present in a cell. These extracts support regulated chromatin assembly, DNA replication, recombination, and repair, and they are not rate limited for the proteins participating in these functions.

Importantly, inhibiting Ku70 activity favors intermolecular joining (Figs. 2 A and 3 A; Labhart 1999). This suggests that multimers are formed by a Ku70-independent pathway reminiscent of what was recently described in mammalian cells (Wang et al., 2003). In *X. laevis*, the Ku70-independent pathway leading to LD and multimer formation is also independent of Mre11, as intermolecular products are still generated in extracts depleted of both Ku70 and Mre11 (Fig. 3 A).

### The kinetics of end-joining reactions is not affected by Mre11 depletion

The aforementioned experiments did not completely rule out that an alternative Mre11-independent NHEJ pathway could substitute for the absence of Mre11. To uncover a hypothetical Mre11-independent pathway, we compared the kinetics of end joining, assuming that different pathways would display different kinetics. We monitored NHEJ activity in Mre11- and mock-depleted extracts at different times during the reaction. NHEJ substrate nonmatching 3′-PSS was incubated in membrane-free egg cytosol, and samples were taken at 0, 0.5, 1, 5, 15, 30, 60, and 120 min. Repaired products were then purified and tested in the colony formation assay. The transforming activity associated with each sample was a read-out of the intramolecular NHEJ activity (circular product formation). At time = 0, no colonies were obtained from either Mre11- or mock-depleted extract samples, thus confirming that all substrate molecules in the input DNA were linear (Fig. 4 A). Repair products were generated as early as 0.5 min in Mre11- and mock-depleted extracts. Furthermore, membrane-free egg cytosol was able to support end joining with similar kinetics in the presence or absence of Mre11. Similar results were obtained when the kinetics of end joining was monitored by Southern blot analysis (Fig. 4 B). We conclude that Mre11 is not required for the rapid NHEJ observed in extracts.

### Mre11 is not required for accurate ligation of DNA ends

Next, we examined the fidelity of end-joining reactions in Mre11- and mock-depleted extracts by sequencing the junctions of cloned NHEJ products. We found that all NHEJ substrates were religated without gross rearrangements (Table I). This is in agreement with a previous study (Pfeiffer and Vielmetter, 1988). The specific joining mode was determined by the terminus configuration of the NHEJ substrate. Ends with cohesive 5′- or 3′-PSSs as well as blunt ends were always religated without net nucleotide loss. Nonmatching 3′-PSS substrates were joined after the alignment of ends that allowed the most efficient base pairing. When no base pairing could occur between the partner ends (blunt end + 5′-PSS, blunt end + 3′-PSS, 5′-PSS + 3′-PSS, and completely nonmatching 5′-PSS), the PSSs were usually preserved by fill-in DNA synthesis. Occasional nucleotide additions, mainly adenosine or thymidine residues, and nucleotide losses, which could be caused by exonucleolytic trimming events, were observed. These events contributed to the variety and complexity of junction formation (Table I). With the exception of a few junction types that occurred very rarely, the spectrum of joining products formed in Mre11-depleted extracts was nearly identical to that obtained in mock-depleted extracts. Moreover, the frequency with which these junction types were formed was not altered in extracts lacking Mre11 protein (Table I). In summary, these data suggest that Mre11 is not required for accurate NHEJ in *X. laevis* egg extracts and does not bias joining toward a specific type of junction.

Altogether, our data demonstrate that Mre11 is dispensable for all aspects of DSB repair by NHEJ: efficiency, kinetics, and fidelity. This is consistent with results in *S. pombe,* in which the loss of *Rad32*, the *Mre11* homologue, or *Rad50* does not impair either the efficiency or the fidelity of NHEJ (Manolis et al., 2001). In contrast, the MRX complex has been reported to be

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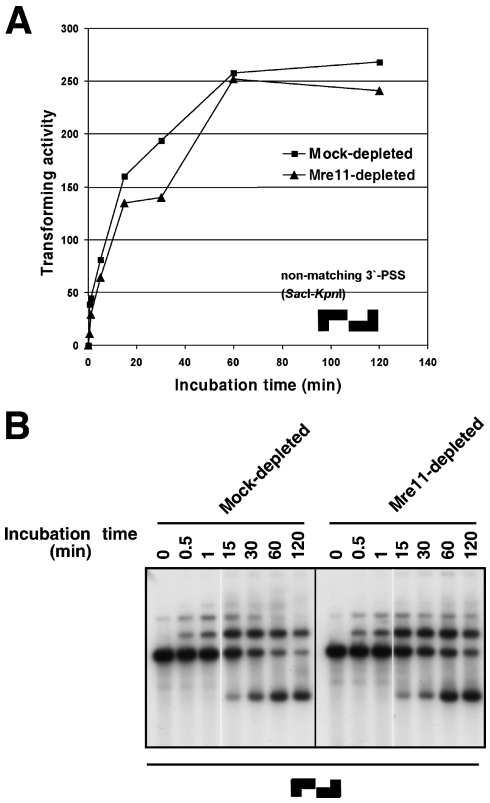


Figure 4. **NHEJ kinetics is not affected by Mre11 depletion.** Nonmatching 3′-PSS substrate SacI-KpnI was incubated in mock- and Mre11-depleted membrane-free cytosol, and samples were taken at the indicated time points. DNA was recovered and analyzed by (A) colony formation assay and (B) Southern blot hybridization (grouping of different segments of the same gel is indicated by dividing lines).

important for NHEJ-mediated repair of both chromosomal and plasmid DSBs in *S. cerevisiae* (Moore and Haber, 1996; Boulton and Jackson, 1998). However, the mechanism by which the MRX complex participates in NHEJ has not been described. The differential requirement for the MRN complex is not a result of the DNA template used because our study and the studies in *S. pombe* and *S. cerevisiae* all used a plasmid-based assay (Boulton and Jackson, 1998; Manolis et al., 2001).

Our results are consistent with the only study addressing the consequences of a complete loss of Mre11 in vertebrates that was performed with a conditional *Mre11*-null chicken DT40 cell line (Yamaguchi-Iwai et al., 1999). Yamaguchi-Iwai et al. (1999) reported a higher radiosensitivity of $MRE11^{-/-}$/$KU70^{-/-}$ cells compared with $MRE11^{-/-}$ cells. They also observed similar levels of radiosensitivity in $MRE11^{-/-}$ $Mre11^{+}$ and $MRE11^{-/-}$ $Mre11^{-}$ cells from G1 to early S phase, when DSBs are repaired mainly by Ku70-dependent NHEJ in this cell line. However, $MRE11^{-/-}$ cells grew very poorly and displayed a high rate of apoptosis upon Mre11 repression. Therefore, end-joining ability in Mre11-deficient cells could not be assessed directly in this study.

In summary, our study establishes that Mre11 is not required for intramolecular end joining of linear DNA molecules or for the Ku70-independent intermolecular end joining of linear DNA in a vertebrate system.

# Materials and methods

### Preparation of NHEJ substrates

Repair substrates for the NHEJ reactions were prepared by digesting pBS II KS plasmid with one or two different restriction enzymes (Table SI). The linearized plasmids were purified by gel extraction, and the completion of digestion was assessed by transformation in *Escherichia coli* DH5α cells (Life Technologies). The absence of single-cut plasmids in double-digested preparations was confirmed after ligation with T4 DNA ligase and subsequent bacterial transformation.

### Immunodepletion

Female *X. laevis* were kept in accordance with Columbia University Institutional Animal Care Usage Committee guidelines. For Mre11 depletion, egg extracts were incubated with protein A–Sepharose beads (GE Healthcare) coupled to anti-XMre11 serum (Mre11 depletion; Costanzo et al., 2001) or preimmune serum/PBS (mock depletion; ratio of extract/beads/serum = 1:0.9:1.1). Anti-XMre11 serum was provided by D. Carroll (University of Utah, Salt Lake City, UT). For Ku70 immunodepletion, egg extracts were incubated with protein G–Sepharose beads (GE Healthcare) coupled to anti-Ku70 antibodies (Ku70 depletion; clone N3H10; Covance) or PBS buffer (mock depletion; ratio of extract/beads/serum = 1:1.1:0.8). For Ku70 and Mre11 double depletion, egg extracts were incubated with a mixture of the two bead preparations. Western blot analysis was performed with 0.5–1 μl of depleted extracts. For Mre11 protein binding to DNA, 20 μl of extracts were incubated with 60 ng/μl of a biotinylated 150-bp DNA fragment bound to Dynabeads M-280 streptavidin (Dynal) for 20 min at 22°C.

### NHEJ assay

A typical NHEJ reaction consisted of 10 μl of immunodepleted extract and 1 μl DNA substrate (1, 10, or 100 ng/μl). Samples were incubated at 15°C for the indicated times, and reactions were stopped by the addition of 10 mM Tris, pH 7.5, 5 mM EDTA, and 1% SDS. 1 mg/ml proteinase K (Roche) was added, and samples were incubated at 50°C for 1 h. DNA was then recovered by organic extraction and ethanol precipitation. NU7026 (Sigma-Aldrich) was dissolved in DMSO and added to the extract before incubation with the DNA substrate.

### Electrophoretic analysis of end-joining products

The DNA was resuspended in $H_2O$/Tris-EDTA buffer, treated with 20 μg/ml RNase A, and electrophoresed on 0.7–1% agarose gels in Tris-borate-EDTA/Tris-acetate-EDTA buffer. SYBR-gold staining (Invitrogen) was performed according to the manufacturer's instructions, and stained gels were scanned using an imager (FluorImager; Molecular Dynamics). Quantification of the signal was performed using ImageQuant software (Molecular Dynamics). Southern blot analysis was performed after standard protocols using radio-labeled pBS KS II sequences as a probe.

### Colony formation assay

20 μl *E. coli* DH5α cell suspension was transformed with DNA recovered from 0.5 μl of cell-free extract (equivalent to 0.5 ng of input substrate) at the end of the incubation time. The number of carbenicillin-resistant colonies/plate defined the value of transforming activity associated with the specific sample.

### Analysis of junctions

After DNA purification from egg extracts, circular end-joining products were cloned in *E. coli* DH5α, and carbenicillin-resistant colonies were selected for the miniscale preparation of plasmid DNA (QIAGEN). Sequencing analysis of cloned junctions was performed by using a DNA sequencer (ABI PRISM 377; Applied Biosystems) and Sequencing Analysis Software 3.4.1 (Applied Biosystems).

### Online supplemental material

Table SI shows the terminus DNA end configuration of the NHEJ substrates used in this study. Fig. S1 shows the characterization of NHEJ reactions by Southern blot hybridization, and Fig. S2 shows NHEJ inhibition by NU7026. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506029/DC1.

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Table I. **Nucleotide sequence and relative frequency of junctions created by joining reactions in mock- (control) and Mre11- (-Mre11) depleted extracts**

| NHEJ substrate type | Terminus sequence | Joining intermediate[a] | Joining product | Control | -Mre11 |
|---|---|---|---|---|---|
| Blunt ends (EcoRV) | 5′-GAT ATC-3′<br>3′-CTA TAG-5′ | 5′-GATATC-3′<br>3′-CTATAG-5′ | 5′-GATATC-3′<br>3′-CTATAG-5′ | 1 (8/8) | 1 (8/8) |
| Cohesive 5′-PSS (EcoRI) | 5′-G AATTC-3′<br>3′-CTTAA G-5′ | 5′-GAATTC-3′<br>●●●●<br>3′-CTTAAG-5′ | 5′-GAATTC-3′<br>3′-CTTAAG-5′ | 1 (8/8) | 1 (7/7) |
| Nonmatching 5′-PSS (NotI–EcoRI) | 5′-C AATTC-3′<br>3′-GCCGG G-5′ | 5′-C▶▶▶▶AATTC-3′<br>3′-GCCGG◀◀◀◀G-5′ | 5′-CGGCCAATTC-3′<br>3′-GCCGGTTAAG-5′ | 0.77 (10/13) | 0.92 (12/13) |
| | | | 5′-CGGCCATTC-3′<br>3′-GCCGGTAAG-5′ | 0.08 (1/13) | 0.08 (1/13) |
| | | | 5′-CGGCCTTC-3′<br>3′-GCCGGAAG-5′ | 0.15 (2/13) | 0 (0/13) |
| Blunt end + 5′-PSS (BamHI–EcoRV) | 5′-G ATC-3′<br>3′-CCTAG TAG-5′ | 5′-G▶▶▶▶ATC-3′<br>3′-CCTAGTAG-5′ | 5′-GGATCATC-3′<br>3′-CCTAGTAG-5′ | 0.92 (11/12) | 0.92 (12/13) |
| | | | 5′-GGATATC-3′<br>3′-CCTATAG-5′ | 0.08 (1/12) | 0.08 (1/13) |
| Cohesive 3′-PSS (PstI) | 5′-CTGCA G-3′<br>3′-G ACGTC-5′ | 5′-CTGCAG-3′<br>●●●●<br>3′-GACGTC-5′ | 5′-CTGCAG-3′<br>3′-GACGTC-5′ | 1 (8/8) | 1 (8/8) |
| Nonmatching 3′-PSS (SacI–KpnI) | 5′-TGGAGCT CCA-3′<br>3′-ACC CATGGGT-5′ | 5′-TGGAGCT CCA-3′<br>●○○<br>3′-ACC CATGGGT-5′ | 5′-TGGAGTACCCA-3′<br>3′-ACCTCATGGGT-5′ | 0.75 (21/28) | 0.63 (17/27) |
| | | | 5′-TGGAGCTACCA-3′<br>3′-ACCTCGATGGT-5′ | 0.11 (3/28) | 0.26 (7/27) |
| | | | 5′-TGGAGCTCCCA-3′<br>3′-ACCTCGAGGGT-5′ | 0.03 (1/28) | 0 (0/27) |
| | | | 5′-TGGAGCTACCCA-3′<br>3′-ACCTCGATGGGT-5′ | 0.03 (1/28) | 0 (0/27) |
| | | | 5′-TGGAGCCCA-3′<br>3′-ACCTCGGGT-5′ | 0.03 (1/28) | 0.07 (2/27) |
| | | | 5′-TGTACCCA-3′<br>3′-ACATGGGT-5′ | 0 (0/28) | 0.04 (1/27) |
| | | | 5′-TGGATACCCA-3′<br>3′-ACCTATGGGT-5′ | 0.03 (1/28) | 0 (0/27) |
| Nonmatching 3′-PSS (PstI–KpnI) | 5′-CTGCA C-3′<br>3′-G CATGG-5′ | 5′-CTGCA C-3′<br>●○●<br>3′-G CATGG-5′ | 5′-CTGTACC-3′<br>3′-GACATGG-5′ | 0.64 (9/14) | 0.64 (9/14) |
| | | | 5′-CTGCACC-3′<br>3′-GACGTGG-5′ | 0.21 (3/14) | 0.28 (4/14) |
| | | | 5′-CTGCC-3′<br>3′-GACGG-5′ | 0.14 (2/14) | 0 (0/14) |
| | | | 5′-CTACC-3′<br>3′-GATGG-5′ | 0 (0/14) | 0.07 (1/14) |
| Blunt end + 3′-PSS (SacI–SmaI) | 5′-GAGCT GGGC-3′<br>3′-C CCCG-5′ | 5′-GAGCTGGGC-3′<br>3′-C◀◀◀◀CCCG-5′ | 5′-GAGCTGGGC-3′<br>3′-CTCGACCCG-5′ | 0.77 (10/13) | 0.71 (10/14) |
| | | | 5′-GAGCTAGGGC-3′<br>3′-C TCGATCCCG-5′ | 0.08 (1/13) | 0.07 (1/14) |
| | | | 5′-GAGCTTGGGC-3′<br>3′-CTCGAACCCG-5′ | 0 (0/13) | 0.14 (2/14) |
| | | | 5′-GAGCTTTGGGC-3′<br>3′-CTCGAAACCCG-5′ | 0 (0/13) | 0.07 (1/14) |
| | | | 5′-GAGCTATAGGGC-3′<br>3′-CTCGATATCCCG-5′ | 0.08 (1/13) | 0 (0/13) |
| | | | 5′-GAAAGGGC-3′<br>3′-CTTTCCCG-5′ | 0.08 (1/13) | 0 (0/13) |
| 5′-PSS + 3′-PSS (PstI–XhoI) | 5′-CTGCA TCGAG$_7$C-3′<br>3′-G C$_7$G-5′ | 5′-CTGCATCGAG$_7$C-3′<br>3′-G◀◀◀◀◀◀◀◀C$_7$G-5′ | 5′-CTGCATCGAG$_7$C-3′<br>3′-GACGTAGCTC$_7$G-5′ | 0.64 (9/14) | 0.71 (10/14) |
| | | | 5′-CTGCTCGAG$_7$C-3′<br>3′-GACGAGCTC$_7$G-5′ | 0.07 (1/14) | 0.07 (1/14) |
| | | | 5′-CTGCACGAG$_7$C-3′<br>3′-GACGTGCTC$_7$G-5′ | 0.07 (1/14) | 0 (0/14) |
| | | | 5′-CTGCAGAG$_7$C-3′<br>3′-GACGTCTC$_7$G-5′ | 0.07 (1/14) | 0 (0/14) |
| | | | 5′-CTGCGAG$_7$C-3′<br>3′-GACGCTC$_7$G-5′ | 0.07 (1/14) | 0 (0/14) |
| | | | 5′-CTG$_5$C-3′<br>3′-GAC$_5$G-5′ | 0 (0/14) | 0.07 (1/14) |
| | | | 5′-CTGCATTCGAG$_7$C-3′<br>3′-GACGTAAGCTC$_7$G-5′ | 0 (0/14) | 0.07 (1/14) |
| | | | 5′-CTGCAGTCGAG$_7$C-3′<br>3′-GACGTCAGCTC$_7$G-5′ | 0 (0/14) | 0.07 (1/14) |
| | | | 5′-CTGCAATCGTG$_7$C-3′<br>3′-GACGTTAGCAC$_7$G-5′ | 0.07 (1/14) | 0 (0/14) |

○, mismatched base-pair; ●, matched base-pair; ▶, fill in DNA synthesis.
[a]The most likely joining intermediate for each NHEJ substrate, as deduced from the analysis of the junction sequences, is indicated.

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