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## Supplemental Data

### The *P. furiosus* Mre11/Rad50 Complex

### Promotes 5' Strand Resection at a DNA Double-Strand Break

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#### Supplemental Experimental Procedures

##### Expression constructs

*Pyrococcus furiosus* Mre11/Rad50 was expressed from a bicistronic pET27b vector (gift from J. Carney). The *P. furiosus* *HerA* and *NurA* genes were amplified from *P. furiosus* genomic DNA (ATCC). The *HerA* and *NurA* genes were cloned separately into pETDuet-1 (Novagen), creating pTP1044 (*HerA*) and pTP1045 (*NurA*) expression constructs. The *RadA* gene was PCR amplified from *P. furiosus* genomic DNA and cloned into pETDuet-1 to create pTP1184. Point mutations were constructed using QuikChange Site-Directed Mutagenesis (Stratagene). Mutations were confirmed by DNA sequencing of the complete open reading frame. Details of construction available upon request.

##### Protein expression and purification

The *Pf*MR complex was coexpressed in BL21 DE3 codonplus *E. coli* as previously described (Hopfner et al., 2000) with minor changes. Briefly, cells were grown to an OD<sub>600</sub> 0.8-1.0 and induced using 0.5 mM IPTG for 4-6 hrs at 37°C. Cells were harvested by centrifugation, flashed frozen in liquid N<sub>2</sub>, and lysed in high salt Nickel A buffer (50 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.0, 500 mM KCl, 2.5 mM imidazole, 10% glycerol, 20 mM β-mercaptoethanol [β-ME]) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5% Tween-20. Cells were lysed using a French Press (Thermo Fisher), sonicated, and insoluble material was pelleted by spinning at 100,000 × g for 1 hr at 4°C. The supernatant was heated at 70°C for 15 min and the precipitated protein was pelleted by spinning at 45,000 × g for 15 min at 4°C. The soluble fraction was loaded onto a Ni-NTA column (Qiagen) in low salt Nickel A buffer (50 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.0, 50 mM KCl, 2.5 mM imidazole, 10% glycerol, 20 mM β-ME) and washed with 10% Nickel B (Nickel A buffer containing 250 mM imidazole). Proteins were resolved with a linear gradient from 10-100% Nickel B buffer. Fractions containing MR were pooled, loaded onto a HiTrap Q HP column (GE) and washed in Buffer A (25 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT)). MR was eluted with 50% Buffer B (25 mM Tris pH 8.0, 1 M NaCl, 10% glycerol, 1 mM DTT). Peak fractions were pooled and mixed with 0.2% Tween-20 before running on a Superdex 200 10/300 GL column (GE) in Buffer A. Fractions containing the MR complex were aliquoted and flash frozen in liquid N<sub>2</sub>. Protein expression and recombinant protein purification for *HerA* and *NurA* was performed similar to the MR protocol with the exception that *NurA* was loaded onto a HiTrap SP HP instead of Q HP. *RadA* expression and purification was performed as previously described (Komori et al., 2000). Mre11, *HerA*, and *NurA* were expressed as N-

terminal 6×His fusion proteins, while Rad50 and RadA were expressed as untagged proteins. Protein concentrations were quantitated using BSA standards on SDS-PAGE gels.

### **Gel filtration**

Wild-type purified HerA (290 nM hexamer), NurA (7 μM monomer), and Mre11/Rad50 (500 nM M<sub>2</sub>R<sub>2</sub>) were incubated as indicated in Buffer A (25 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol) at 55°C for 15 min. Tween-20 was added to 0.1% before resolving on a Superdex 200 PC 3.2/30 column run in Buffer A. Fractions were run on 10% SDS-PAGE gels, transferred to PVDF membranes (Millipore), and probed with α-His antibodies conjugated to IRDYE-800 dye (Rockland). Western blots were analyzed on a Licor Odyssey system.

### **Oligonucleotide DNA substrates**

The substrates in Fig. 5A were constructed as follows: Substrate 1 consisted of 5' [<sup>32</sup>P]-labeled TP74 (5'-CTGCAGGGTTTTTGTTCAGTCTGTAGCACTGTGTAAGACAGGCCA-3') annealed to TP124 (5'-CATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG-3'). Substrate 2 consisted of 5' [<sup>32</sup>P]-labeled TP124 annealed to TP125 (5'-CTGCAGGGTTTTTGTTCAGTCTGTAGCACTGTGTAAGACAGGCCAGATG-3'). Substrate 3 consisted of 5' [<sup>32</sup>P]-labeled TP580 (5'-CTGCAGGGTTTTTGTTCAGTCTGTAGCACTGTGTAAGACAGGCCsAsGsAsTsG-3') annealed to TP124. Phosphorothioate bonds are indicated by “s” between the nucleotides. Substrate 4 consisted of 5' [<sup>32</sup>P]-labeled TP694 (5'-AAGTAAATCATTAATCTAACAATGCGCTCATCGTCsAsTsCsCsT-3') annealed to TP695 (5'-AGGATGACGATGAGCGCATTGTTAGATTAATGATTsTsAsCsTsT-3'). The oligonucleotide substrate in Fig. S4A consisted of 3' [<sup>32</sup>P]-labeled TP124 annealed to TP580. The helicase assay substrate in Fig. S4C was constructed by annealing 5' [<sup>32</sup>P]-labeled TP1760 (5'-CCACTACGTGAACCATCACCCAAATCAAGTTTTTTGGGGTTCGAGGTGC-3') to M13 single-stranded DNA. 3' or 5' [<sup>32</sup>P]-labeling was performed as described below.

### **Preparation of oligonucleotide and DNA substrates**

Oligonucleotide DNA substrates (MWG) were either 3' end-labeled with [ $\alpha$ -<sup>32</sup>P]cordycepin (3' deoxyadenosine) (NEN) and Terminal deoxytransferase (TdT) (Roche) or 5' end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (NEN) and T4 Polynucleotide Kinase (PNK) (NEB) and annealed to a molar excess of unlabeled complementary oligonucleotide. The 2.5 kb internally-labeled substrate was generated by PCR using [ $\alpha$ -<sup>32</sup>P]dTTP (NEN) and gel purified by native agarose gel electrophoresis and electroelution. The helicase assay substrate consisted of a 5' [<sup>32</sup>P]-labeled 48 nt oligonucleotide annealed to M13 single-stranded DNA (NEB). The 5' Biotin/bead substrate was constructed by ligating an oligonucleotide containing a 5' Biotin-TEG (MWG) to the plasmid DNA substrate and attaching the DNA to streptavidin-coated magnetic beads (Dynal). The beads were washed extensively to remove unbound DNA. Labeled linear dsDNA for the joint-molecule assay was prepared by PCR amplification of a 415 bp region of M13 phage DNA in the presence of [ $\alpha$ -<sup>32</sup>P]dTTP and gel purification by native gel electrophoresis and electroelution. Labeled linear DNA for strand invasion experiments was prepared by PCR amplification of a 2.2 kb region of pTP179 in the presence of [ $\alpha$ -<sup>32</sup>P]dATP (NEN), which was digested at both ends with SphI (NEB) to produce a 1.4 kb linear DNA fragment and gel purified by native gel electrophoresis. Supercoiled 3.6 kb pTP179 plasmid DNA was prepared by sucrose gradient centrifugation.

### **Helicase assays**

Helicase reactions contained 0.15 nM [<sup>32</sup>P]-labeled oligonucleotide/ssM13 DNA complexes in 20 µl reactions containing 20 mM HEPES pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM ATP, 50 mM NaCl, 1 mM DTT, and 100 µg/ml BSA. Reactions were incubated at 55°C for 1 hr before separation by native agarose electrophoresis in 0.8% TAE agarose gels for 1 hr at 3.5 V/cm. Reactions were terminated by the addition of 0.2% SDS, 10 mM EDTA, and 0.5 µg/ml Ethidium bromide (EtBr). Gels were dried and products were analyzed by phosphorimager (Biorad or GE). See above for oligonucleotide sequences and preparation of the duplex substrate.

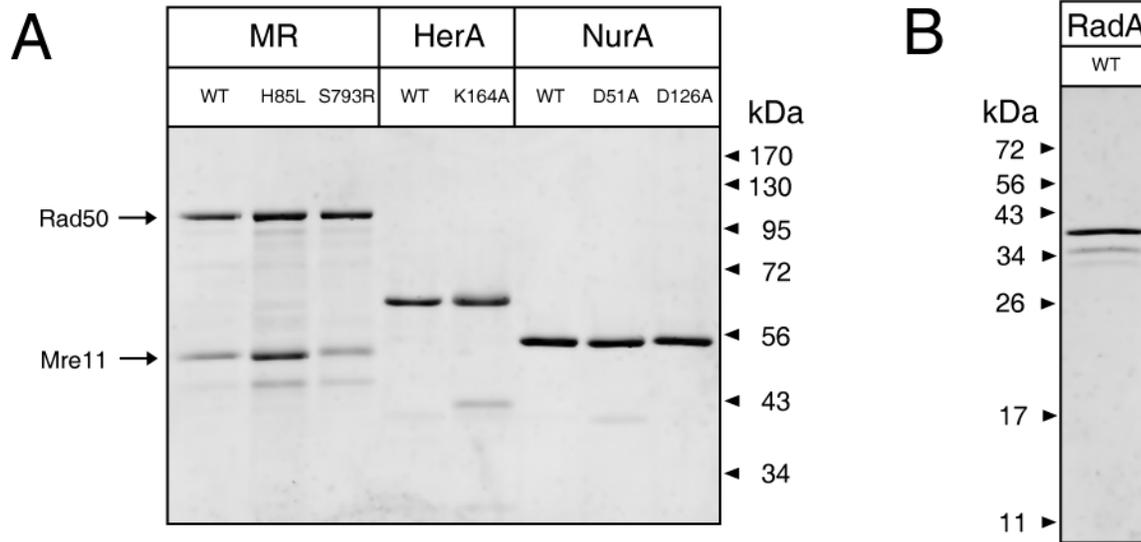
### **ATPase assays**

Wild-type or K164A HerA was incubated in 10 µl reactions containing 25 mM MOPS pH 7.0, 5 mM MgCl<sub>2</sub>, 40 mM NaCl, 2 mM DTT, and 50 µM [ $\gamma$ -<sup>32</sup>P]ATP (MP Biomedical) at 65°C for 1 hr in the presence or absence of 100 ng linear double-stranded DNA (1 kb ladder, Invitrogen). Reactions were stopped by the addition of 0.2% SDS, 10 mM EDTA, and 0.5 µg/ml EtBr. Reactions were analyzed by thin-layer chromatography (TLC) as described previously (Bhaskara et al., 2007).

### **Processing of gels and membranes for Southern hybridization**

Agarose gels were washed into 20× SSC (3 M NaCl, 0.3 M sodium citrate) and DNA was transferred by capillary action onto nylon membranes (NEN) overnight in 20× SSC. Membranes were probed with RNA complementary to either the 3' or 5' strand of the DNA substrate in a 1 kb region on one end, adjacent to the SacI site. The probes were internally labeled with [ $\alpha$ -<sup>32</sup>P]CTP (NEN) and were made using Riboprobe System T7 (Promega) according to the manufacturer's instructions. The 3' strand-specific probe of a 1 kb region adjacent to the bead attachment site was made by primer extension with [ $\alpha$ -<sup>32</sup>P]dATP (NEN). Denatured DNA controls consisted of substrate DNA denatured with NaOH.

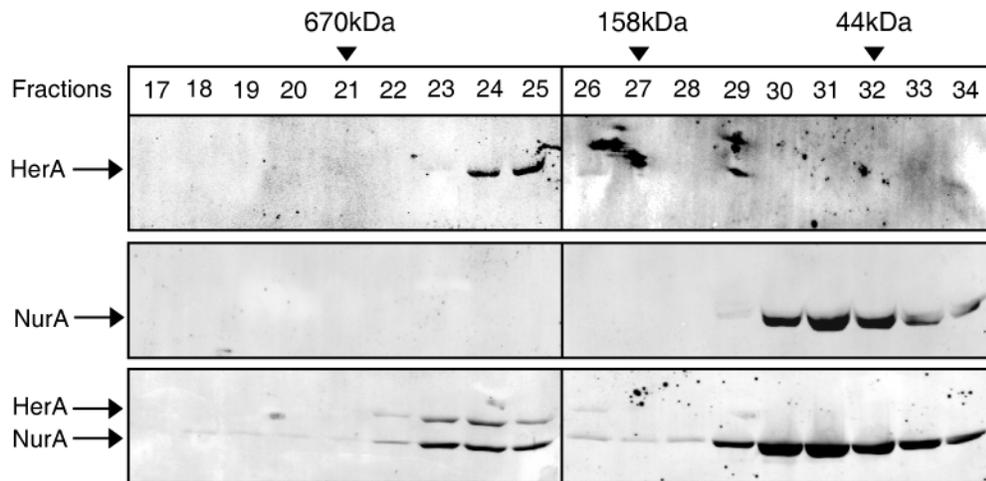
Supplemental Figures



**Figure S1. Protein gels of purified recombinant *P. furiosus* Mre11/Rad50, HerA, NurA, and RadA.**

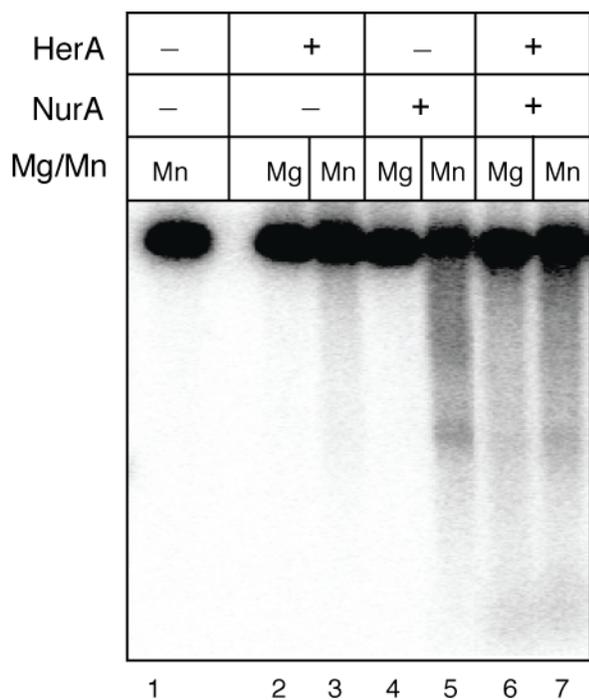
(A) 0.5  $\mu$ g wild-type and mutant MR, HerA, and NurA run on 10% SDS-PAGE gels as indicated.

(B) 1  $\mu$ g wild-type RadA run on 16% SDS-PAGE gels. Migration of molecular weight markers are indicated. Gels were stained with Colloidal Blue Coomassie stain (Invitrogen).



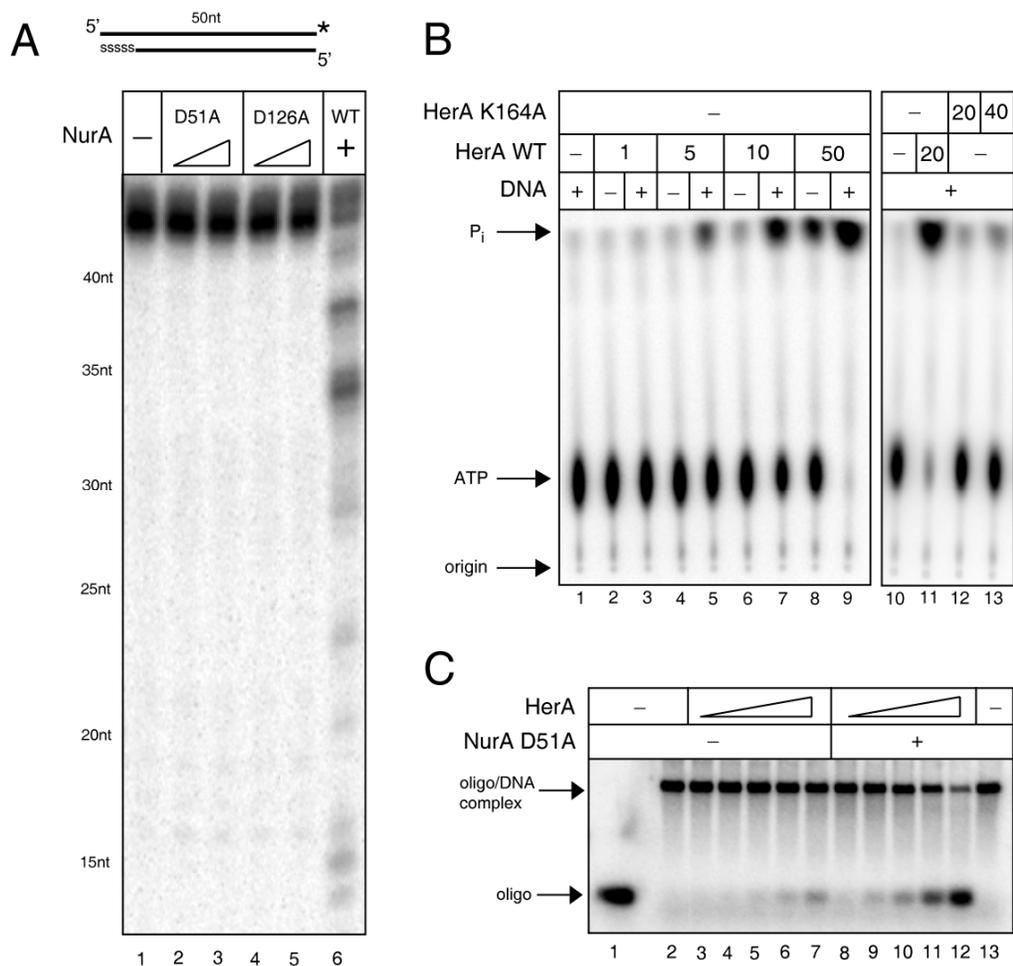
**Figure S2. *P. furiosus* NurA and HerA interact physically.**

Superdex 200 gel filtration of purified HerA and NurA. Purified proteins were incubated alone (HerA: top panel, NurA: middle panel) or in combination (bottom panel) at 55°C for 15 min before gel filtration, as indicated, and fractions were analyzed by SDS-PAGE and  $\alpha$ -His western blot. Migration of molecular weight markers are indicated.



**Figure S3. *P. furiosus* HerA facilitates NurA activity in magnesium.**

Nuclease assays were performed using 2.5 kb internally [<sup>32</sup>P]-labeled dsDNA. Reactions contained 13.4 nM wild-type HerA, 96.2 nM wild-type NurA, 80 mM NaCl, 1 mM ATP, and 5 mM MgCl<sub>2</sub> or MnCl<sub>2</sub> as indicated. Reactions were incubated at 65°C for 1 hr before separation on a native agarose gel.

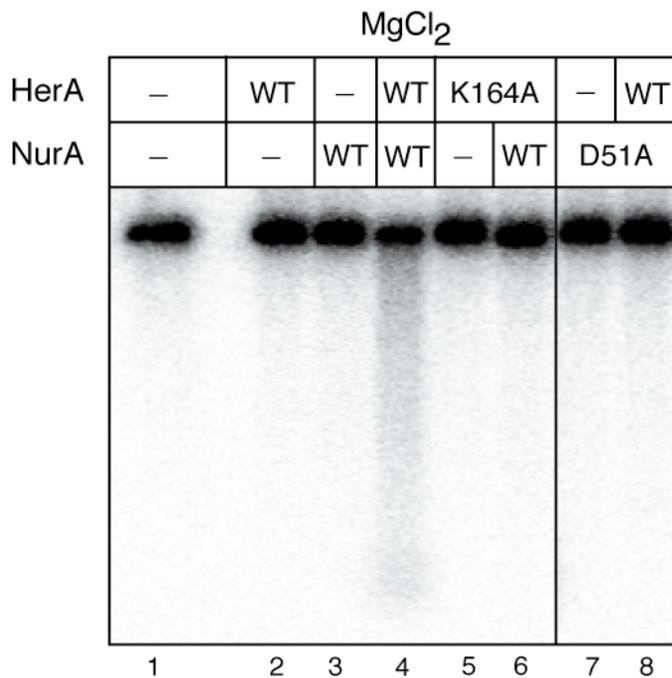


**Figure S4. Biochemical characterization of NurA and HerA.**

(A) Exonuclease assays were performed with 190 nM wild-type NurA (+), 190 or 960 nM D51A, and 190 or 960 nM D126A NurA, as indicated. The 50 bp double-stranded oligonucleotide substrate was 3' [<sup>32</sup>P]-labeled on the top strand and contained 5 phosphorothioate bonds (“sssss”) on the 3' end of the bottom strand. Reactions included 4 nM substrate, 60 mM NaCl, and 1 mM MnCl<sub>2</sub> and were carried out at 65°C for 1 hr. Reaction products were analyzed on 15% denaturing polyacrylamide sequencing gels. Migration of single-stranded DNA markers are indicated.

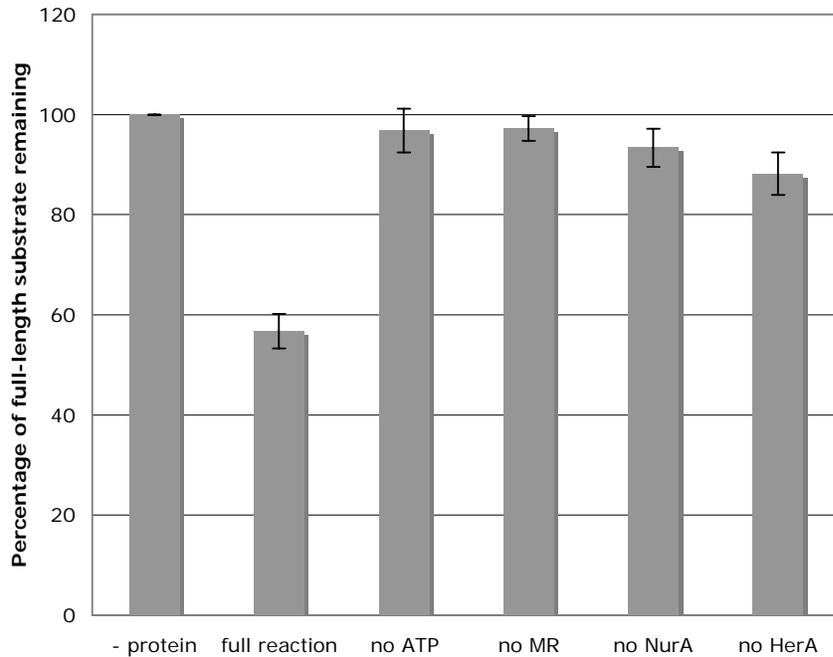
(B) HerA ATPase assays were performed with 1-50 nM wild-type or 20-40 nM K164A HerA and 100 ng dsDNA as indicated using [<sup>32</sup>P]ATP. Reactions were incubated at 65°C for 1 hr and reaction products were analyzed by TLC. The analogous mutation (K153A) was shown to inhibit helicase activity in *S. acidocaldarius* HerA (Constantinesco et al., 2004). Positions of the origin, ATP, and inorganic phosphate (P<sub>i</sub>) are indicated.

(C) Wild-type HerA helicase assays were performed with a 5' [<sup>32</sup>P]-labeled 48 nt oligonucleotide/M13 ssDNA duplex. Reactions contained 0.3, 0.7, 1.3, 2.7, or 6.2 nM wild-type HerA and 9.6 nM NurA D51A as indicated. Reactions were incubated at 55°C for 1 hr before separation on native 0.8% TAE agarose gels. Lane 1 contains boiled substrate. The positions of the oligonucleotide/ssM13 complex and oligonucleotide are indicated.



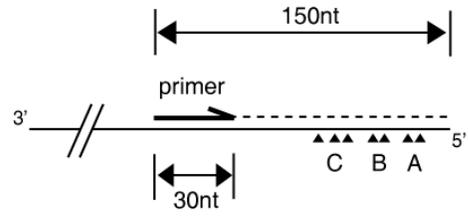
**Figure S5. Cooperative DNA degradation by HerA and NurA require catalytically active enzymes.**

Nuclease assays were performed using 2.5 kb internally [<sup>32</sup>P]-labeled dsDNA. Reactions contained 13.4 nM wild-type HerA, 96.2 nM wild-type NurA, 5 mM MgCl<sub>2</sub>, 1 mM ATP, and 100 mM NaCl. HerA K164A and NurA D51A mutant proteins were included as indicated. Reactions were incubated at 65°C for 1 hr before separation on a native agarose gel.

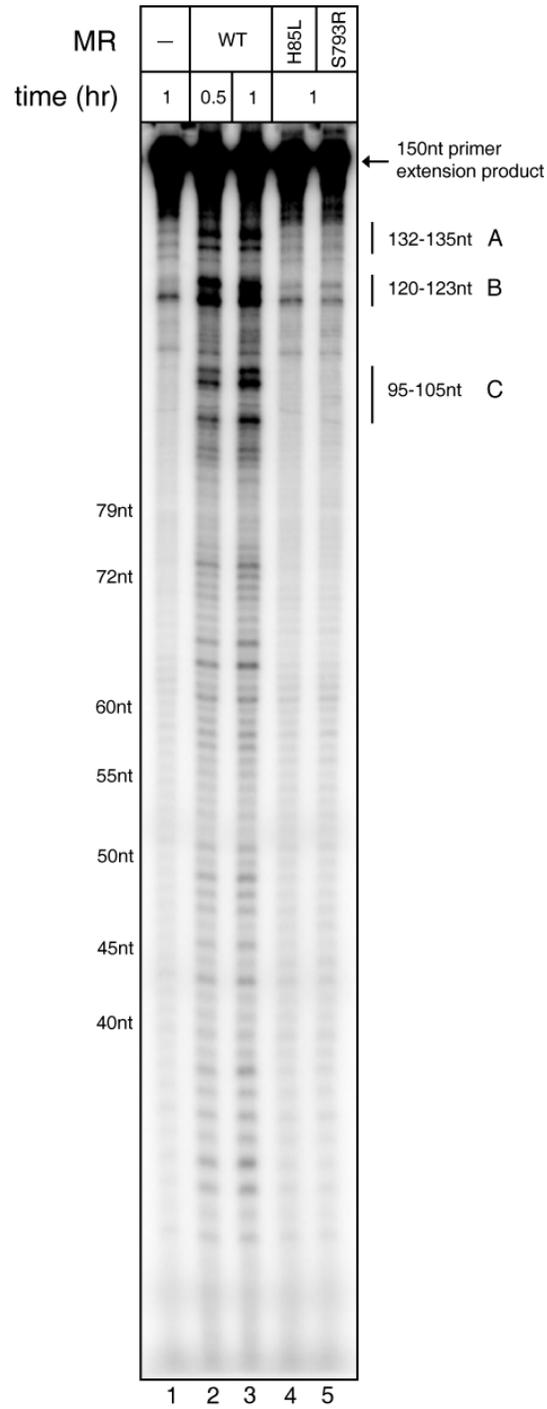


**Figure S6. Quantitation of DNA degradation by *P. furiosus* Mre11/Rad50/HerA/NurA.** Five independent sets of reactions containing the components indicated above were analyzed as described in Fig. 1 and the percentage of the full-length linear substrate remaining after the reaction was quantitated using phosphorimager analysis. The amount of linear DNA in the “- protein” lane in each experiment was set to 100%. The error bars are calculated from standard deviation. The full reaction contains pfMre11/Rad50, HerA, NurA, [<sup>32</sup>P]-labeled linear DNA, ATP, and magnesium chloride.

**A**



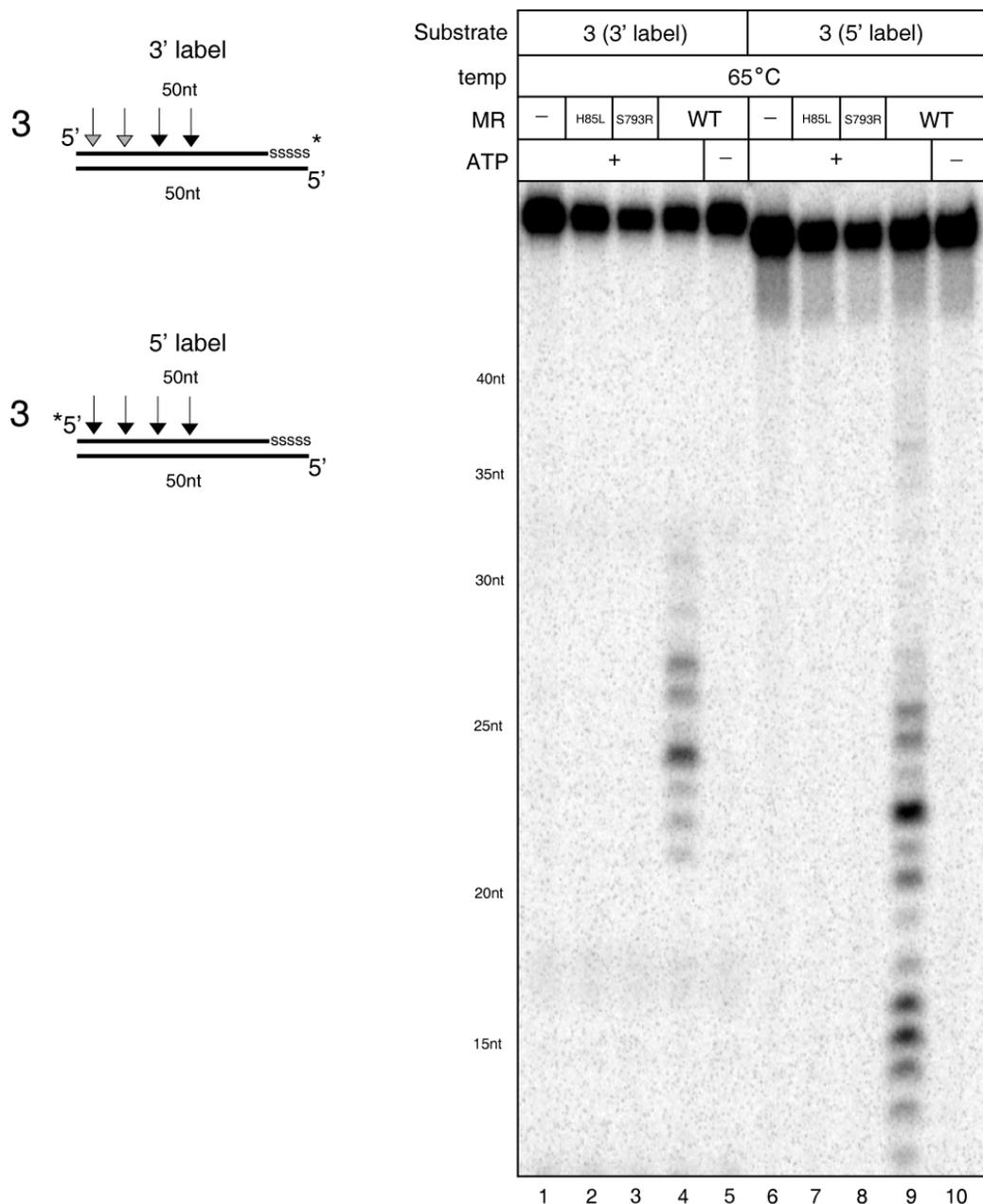
**B**



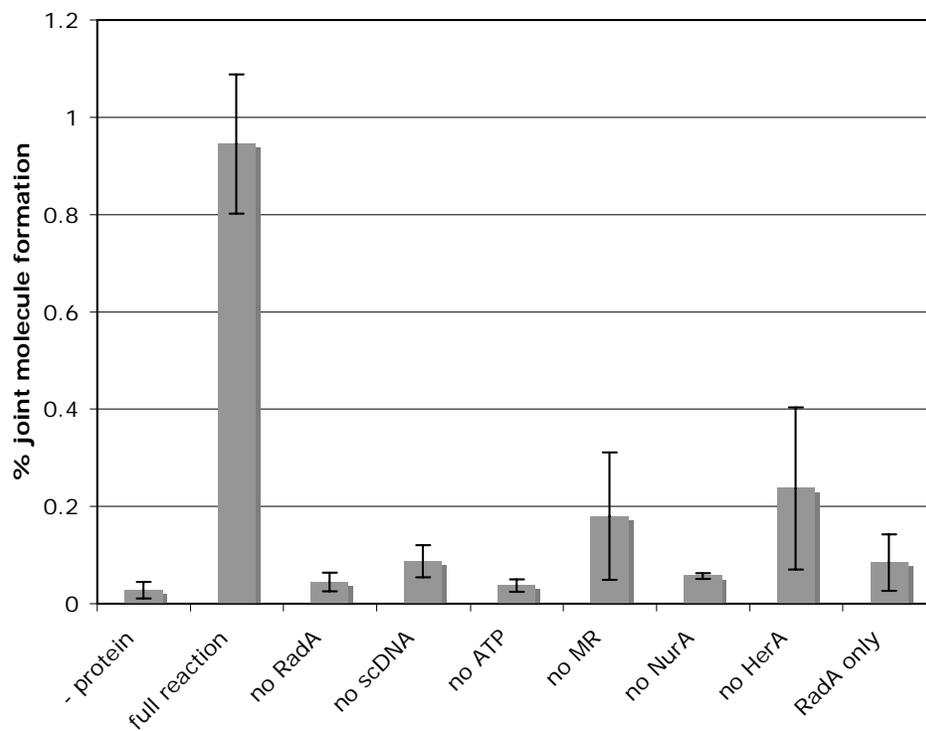
**Figure S7. Primer extension to determine length of DNA resected by *P. furiosus* Mre11/Rad50.**

(A) Schematic of primer extension reaction. For simplicity, only the bottom strand of pTP163 is shown. The lengths of the full-length primer extension product and primer are indicated. The internally labeled primer extension product is indicated by the dashed line. Major MR resection sites are indicated as arrow heads and grouped into A, B, and C subgroups containing 2 or 3 cut sites.

(B) *P. furiosus* Mre11/Rad50 resection reactions were performed with 2.3 nM pTP163 linearized with SacI and 165 nM Mre11/Rad50 at 65°C for 30 min or 1 hr as indicated. Reactions were then used in primer extension reactions and the primer extension products were analyzed on 10% polyacrylamide denaturing sequencing gels. The position of the 150 nt primer extension product and the various resected products are indicated. Single-stranded DNA markers are indicated.



**Figure S8. *P. furiosus* Mre11/Rad50 performs endonucleolytic cleavage of double-strand DNA oligonucleotide duplexes.** Substrate 3 from Figure 5 was [ $^{32}$ P]-labeled on the 3' end of the top strand or on the 5' end of the top strand. Reactions were performed with pfMR wild-type, Mre11-H85L, or Rad50-S793R mutant complexes as indicated and analyzed as described for Fig. 5. All reactions were performed in the presence of 5 mM magnesium chloride. The positions of the nucleolytic cuts are shown with vertical arrows in the diagrams at left. The cleavage sites shown in gray arrows are not visible with the 3' labeled-substrate, thus the internal cleavage events must occur first or always coincident with the cleavage events near the 5' end of the top strand.



**Figure S9. Quantitation of Joint Molecule Formation by *P. furiosus***

**Mre11/Rad50/HerA/NurA/RadA.** Five independent sets of reactions containing the components indicated above were analyzed as described in Fig. 6 and the percentage of the linear substrate converted into joint molecules (D-loop products) was quantitated using phosphorimager analysis. The error bars are calculated from standard deviation. The full reaction contains pfMre11/Rad50, HerA, NurA, RadA, [<sup>32</sup>P]-labeled linear DNA, supercoiled plasmid DNA, ATP, and magnesium chloride.

## Supplemental References

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