Supplementary Materials and Methods

**DNA substrates**

PAGE-purified DNA substrates were purchased from Bioneer Co. (Daejeon, Korea). For crystallization, DNA molecules were further purified and annealed. Crystal I of the DNA substrate contained 5'-ACGTGCTTACACACAGGGTTAGGGAACCTT-3', 5'-CAAGTTCACCCTAACC TGAC-3', and 5'-CTGTGTGGAAGCAGC-3'. Crystal II of the DNA substrate contained 5'-TAGACACACATTGGGACATGCAG-3', 5'-TCTGATGTAGTCATT-3', and 5'-GAATGTGTGTCT-3'. Crystal III of the DNA substrate contained 5'–GAATGTGTGTCTCTCAATC-3', 5'-GGATTG-3', and 5'–TAACCAGACACACATT-3'. The nHJ substrates for the nuclease assay F60: 5’–GACGCTGCGACTTCTACCATGGCCTTG

CTAGGACATCTTGGCCACCTGCTTGTCTACC–3', 5'-32P-labeled R60: 5'–ATCGATAGT CG GATACTCGTCACAGCTCCATGGCAAGGACTGGTAGAAGTGCGGCAGGGT–3', RF1: 5'-GGGTGAACAGGGTTAGGGCAGATGATC-3', nHJL: 5'-CAGATGCCAGCTTGACTGACTGACTCCAGAAGAATGTCC-3', and nHJS: 5'-TCTG GGCGGGAATAGGCACGTGTAATTACGCCAGACATGGAGCTGTGACGAGTATCCGACTATCGA-3'. 5'-32P-labeled substrates were prepared with Poly nucleotide kinase (Roche Applied Science, Rockford, IL, USA) and γ-32P-dATP (PerkinElmer, Waltham, MA, USA). The substrates were annealed in buffer containing 150 mM NaCl and, 15 mM sodium citrate buffer, boiled at 95°C for, 5 min, and then allowed to cool. The 3'flap DNA substrate was prepared by annealing 5'-32P-labeled R60, nHJL, and nHJS.

DNA substrates of FRET were purchased from Integrated DNA Technologies, Inc. (Leuven, Belgium). A 3' flap DNA substrate contained 5'-FAM-TTTACGAATGTGTAAC-3', 5'-GTCT GGTTAGGGA-3', and 5'-TMR-TCCCTAACCAGACGACGTAATTACGCCAGAC-3'. A double strand DNA substrate contained 5'-FAM-TTTACGAATGTGTAACGTCTGGTTAGGGA-3', and 5'-TMR-TCCCTAACCAGACGACGTAATTACGCCAGAC-3'. The substrates were annealed using the same method described above.

**Mutagenesis**

All the hMus81 and hEme1 mutants used in this study were created by PCR-based methods (Stratagene). The mutant proteins were purified using affinity chromatography followed by Resource-S as described above. For the EHhH2 mutant, Arg534 and Thr541 of hEme1 were simultaneously mutated to glutamic acid and tyrosine. Arg491 and Ser493 of hEme1 were simultaneously mutated to glutamic acid and tryptophan. MHhH2 mutants, Arg483, Lys489, and Arg530 of hMus81 were mutated to alanine. For the wedge mutant, Ile 344 and Ile 345 of
hMus81 were mutated to arginine. 5' end binding pocket mutant, Thr383 and Ala387 of hMus81 were mutated to arginine. Arg348 and Arg355 of hMus81 were simultaneously mutated to glutamic acid. For the active site mutants, Asp274, Glu277, and Asp307 of hMus81 were mutated to alanine, respectively.

**Fluorescence measurements and FRET calculations**

To examine possible DNA conformation changes, a fixed amount (0.5 μM) of FAM- and/or TMR-labeled DNA was titrated with various amounts of hMus81-Eme1 (0.5–2.5 μM) and incubated for 5 min in buffer containing 20mM BTP-HCl, pH 7.0, 0.2M NaCl, 1mM b-mercaptoethanol, and 5% glycerol. The samples were directly excited at 490 nm and emission signals were collected at 510-640 nm. Background fluorescence caused by direct excitation of TMR (acceptor) at 490 nm was measured in a separate titration reaction and subtracted from the values of samples. Fluorescence was measured using a Cary Eclipse Fluorescence spectrophotometer (VARIAN Australia, Inc.). Steady-state fluorescence experiments were performed with the spectrofluorometer thermostat set at 20°C. More than three independent FRET experiments were performed.