Materials and Methods

Mn^{2+}-dependent endonuclease assay

Unless indicated otherwise, the Mn^{2+}-dependent endonuclease reactions were carried out at 37°C for 40 minutes in 40-μl mixtures that contained 20 mM HEPES-NaOH (pH 7.4), 3 mM $MnSO_4$, 0.5 mM ATP, 1 mM DTT, 0.2 mg/ml BSA, 70 mM NaCl, and 0.9 nM f1MR59 supercoiled DNA (1). When indicated the reaction mixtures included MutLγ (6-44 nM), MutLγ-D1223N (6-44 nM), or MutLα (22-176 nM). Some of the indicated Mn^{2+}-dependent endonuclease reactions were carried out in the presence of MutSβ (13 nM). These Mn^{2+}-dependent endonuclease reactions were performed as described above except that 70 mM NaCl was replaced with a mixture of 55 nM NaCl and 15 mM KCl. The endonuclease reactions were terminated by the addition of SDS, EDTA and proteinase K to 0.1%, 30 mM, and 4 mg/ml, respectively. The mixtures were incubated at 50°C for 20 min, and the protein digestion was stopped by the addition of PMSF to 0.6 mM. The DNA products were separated on 0.8% native agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2), followed by staining of the gels with 0.6-μg/ml ethidium bromide. The images were captured with a cooled CCD camera, and the DNA species were quantified with an ImageJ software.

Mg^{2+}-dependent endonuclease assay

The Mg^{2+}-dependent endonuclease reactions were performed at 37°C for 40 min in 40-μl mixtures that contained 20 mM HEPES-NaOH (pH 7.4), 116 mM KCl, 24 mM NaCl, 3 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 0.4 mg/ml BSA, indicated proteins, and 50 ng indicated relaxed ccDNA. When present, MutLγ, MutLγ-D1223N, MutSβ, PCNA, and RFC concentrations were 39 nM, 39 nM, 25 nM, 15 nM, and 1.3 nM, respectively. The reactions were stopped by the addition of a 30-μl mixture containing 0.35% SDS, 0.4 M NaCl, 13 mM EDTA, 0.7 mg/ml proteinase K, and 2 mg/ml glycogen, and then incubated at 50°C for 20 min. The DNA products were

extracted with a 1:1 mixture of phenol/chloroform and precipitated with isopropanol. The recovered DNAs were cleaved with BanI or ScaI, separated in 1.2-1.4% agarose gels in 40 mM NaOH/2 mM EDTA, transferred onto nylon membranes, and analyzed by Southern hybridizations with ^{32}P-labeled probes. The indirectly labeled DNA species were visualized with a Typhoon biomolecular imager (GE HealthCare) and quantified with an ImageQuant software. During quantification, the background value of DNA degradation present in the control reaction carried out in the absence of human proteins was subtracted from the value of DNA degradation in the indicated reaction.

ATPase assay

The ATPase reactions were carried out in 40-µl mixtures that contained 20 mM HEPES-NaOH (pH 7.6), 140 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.2 mg/ml BSA, 0.5 mM ATP, 3.75 µCi/ml [γ-^{32}P]ATP (6000 Ci/mmol) (Perkin Elmer), and 0.19 µM MutLγ or 0.19 µM MutLγ-D1223N. The reactions were incubated at 37°C for indicated time. The reactions were stopped by the addition of SDS and EDTA to 0.2% and 23 mM, respectively. The products of the ATPase reactions were resolved by thin layer chromatography in 0.125 M LiCl/0.45 M CH_3COOH on PEI plates (Merck). The ^{32}P-labeled species were visualized with a Typhoon biomolecular imager (GE HealthCare) and quantified with an ImageQuant software. During quantification, phosphorimager data were corrected for the background level of free ^{32}P present in the preparation of [γ-^{32}P]ATP.

DNA expansion assay

DNA expansion reactions were carried out at 37°C for 40 min in 80-µl mixtures containing 20 mM HEPES-NaOH (pH 7.4), 18 mM NaCl, 92 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.2 mg/ml BSA, 0.1 mM each dATP, dGTP, dCTP, and dTTP, 240 µg cytosolic H6 cell extract, and

0.6 nM of 3-nt loop-containing relaxed ccDNA. When indicated, the DNA expansion reaction mixture was supplemented with purified human protein(s). If MutSβ, MutLγ, MutLγ-D1223N, and MutLα were present in the DNA expansion reaction mixture, their concentrations were 13 nM, 29 nM, 29 nM, and 29 nM, respectively. The reactions were stopped by the addition of a 60-μl mixture containing 0.35% SDS, 0.4 M NaCl, 13 mM EDTA, 0.3 mg/ml proteinase K, and 2 mg/ml glycogen, followed by incubation of the mixtures at 50°C for 15 min. The DNA products were extracted with a 1:1 phenol/chloroform mixture and precipitated with isopropanol. The recovered DNA products were hydrolyzed with BanI and BmtI to score 3-nt expansion or with BanI and HindIII to score the removal of the 3-nt loop. The cleaved DNA products were separated on 1.3% native agarose gels in 1x TAE buffer, transferred onto nylon membranes in 0.2 M NaOH, and analyzed by Southern hybridizations with a ^{32}P-labeled oligonucleotide (5′-GACAGTTACCAATGCTTAATCAGTG-3′). The DNA species were visualized with a Typhoon biomolecular imager (GE HealthCare) and quantified with an ImageQuant software. During quantification, the background level of the double restriction endonuclease cleavage of DNA products of the control reaction carried out in the absence of the cell extract and human proteins was subtracted from the value of the double restriction endonuclease cleavage of DNA products of the indicated reaction.

1. Dzantiev L, *et al.* (2004) A defined human system that supports bidirectional mismatch-provoked excision. *Mol. Cell* 15:31-41.

Supplementary Figures

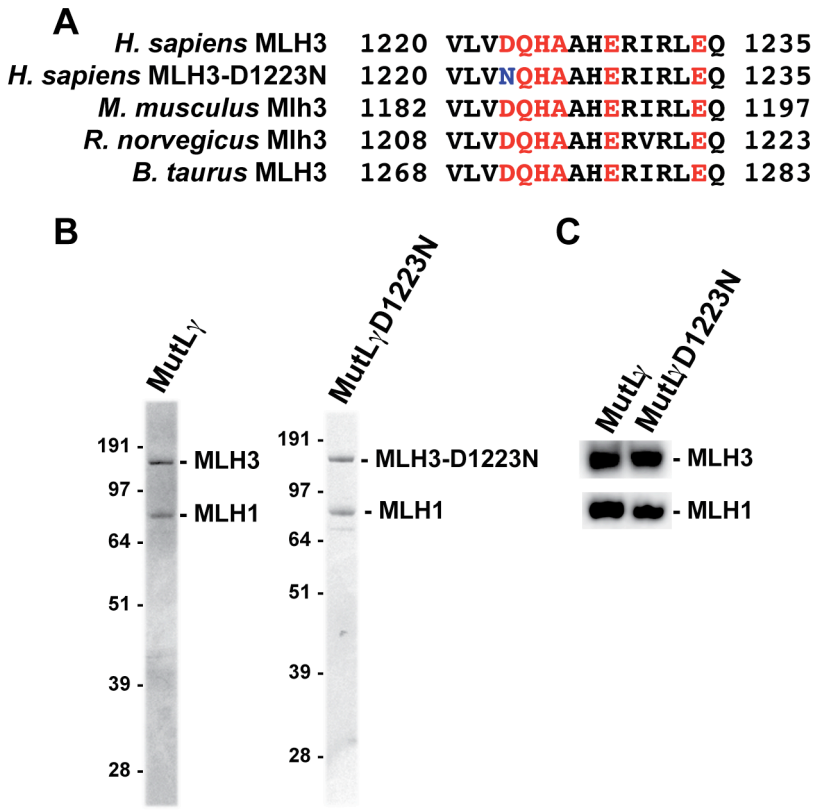

Figure S1. Purification of human MutLγ and MutLγ-D1223N. (**A**) The DQHA(X)$_2$E(X)$_4$E endonuclease motif is preserved in mammalian MLH3 proteins. Please note that this motif is disrupted in the human MLH3-D1223N mutant. (**B**) SDS-PAGE analysis of human MutLγ and MutLγ-D1223N that were expressed in and purified from insect Sf9 cells. The protein bands were visualized by Coomassie R-250 staining. (**C**) The purified MutLγ and MutLγ-D1223N are recognized by α-MLH1 and α-MLH3 antibodies in a Western blot analysis.

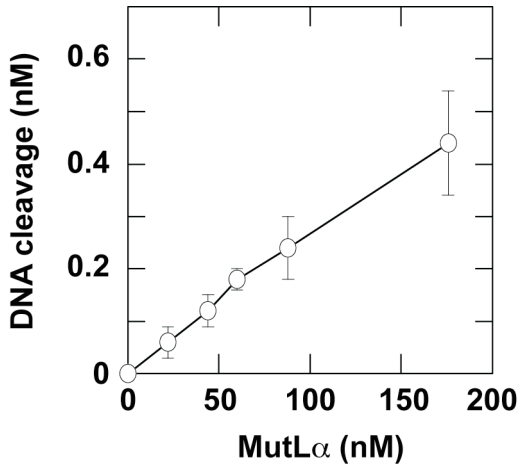

Figure S2. Mn^{2+}-dependent endonuclease activity of human MutLα. The Mn^{2+}-dependent endonuclease reactions that occurred in the presence of the indicated concentrations of MutLα were carried out and analyzed as described in **Fig. 1A**.

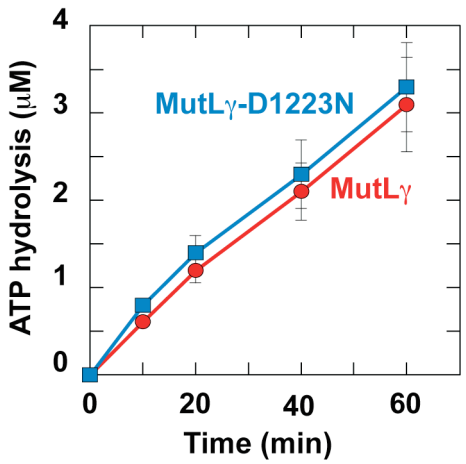

Figure S3. ATPase activities of MutLγ and MutLγ-D1223N. The ATPase reactions were carried out in the presence of MutLγ or MutLγ-D1223N for the indicated time. The other conditions and analysis are described under Materials and Methods. The data are shown as averages ± 1 S.D. (n = 4) and were obtained using the same preparations of proteins.

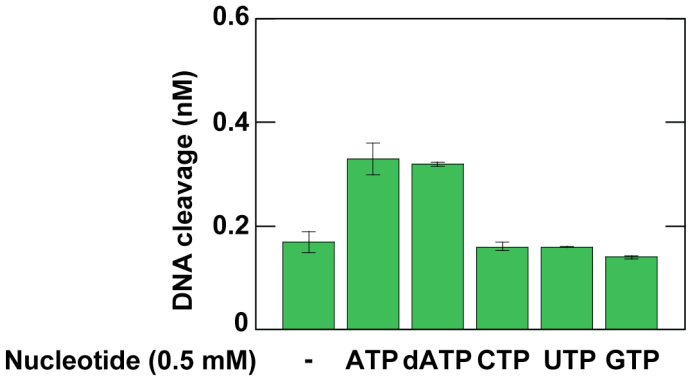

Figure S4. The effects of different nucleotides on Mn^{2+}-dependent endonuclease activity of human MutLγ. The endonuclease reactions that took place in the presence of 22 nM MutLγ and 3 mM $MnSO_4$ were carried out and analyzed as described in **Fig. 1A** except that 0.5 mM ATP was absent or replaced with 0.5 mM dATP, CTP, UTP, or GTP as indicated. The data shown are averages ± 1 S.D. ($n \geq 2$).

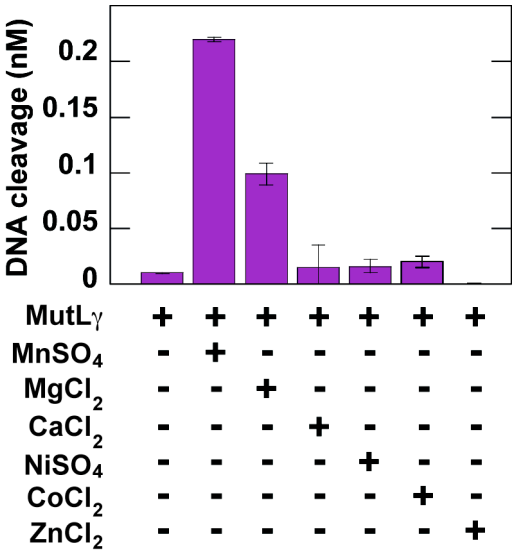

Figure S5. Endonuclease activity of human MutLγ in the presence of different divalent metal cations. Endonuclease reactions in the presence of 22 nM MutLγ, 0.5 mM ATP, and 1 mM divalent cations were performed and analyzed as described in **Fig. 1A** except that 3 mM $MnSO_4$ was absent or replaced with 1 mM $MnSO_4$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $NiSO_4$, 1 mM $CoCl_2$, or 1 mM $ZnCl_2$ as indicated. The data shown are averages ± 1 S.D. ($n \geq 2$).

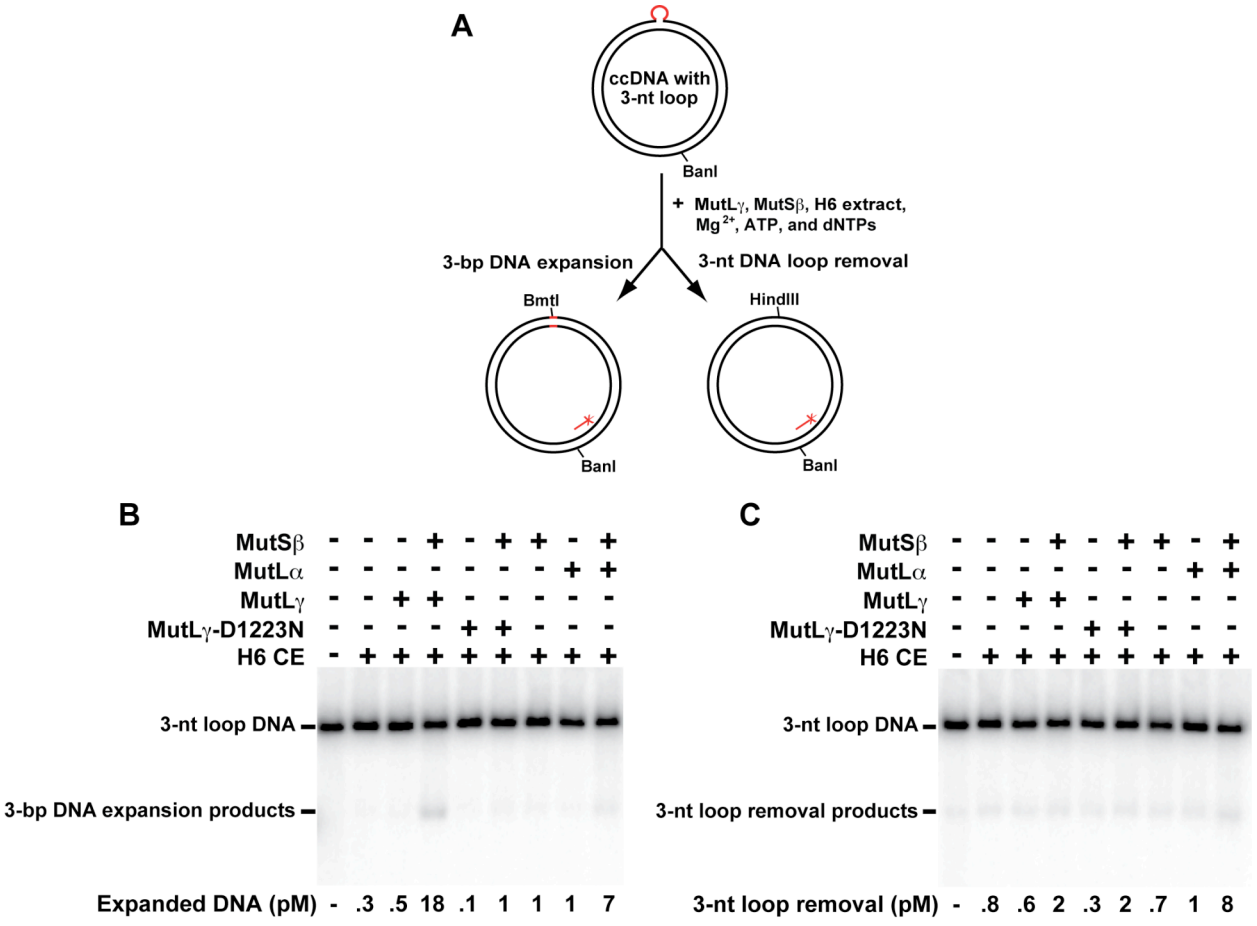

Figure S6. Human MutLγ endonuclease- and MutSβ-dependent DNA expansion in the H6 extract system. (**A**) Outline of the experiments. 3-bp DNA expansion (**B**) and 3-nt loop removal (**C**) in human *MLH1*$^{-/-}$ *MSH3*$^{-/-}$ H6 cell extracts that were supplemented with MutLγ, MutSβ, ATP, and Mg^{2+}. Reactions were performed and analyzed by Southern blot as described under Materials and Methods.

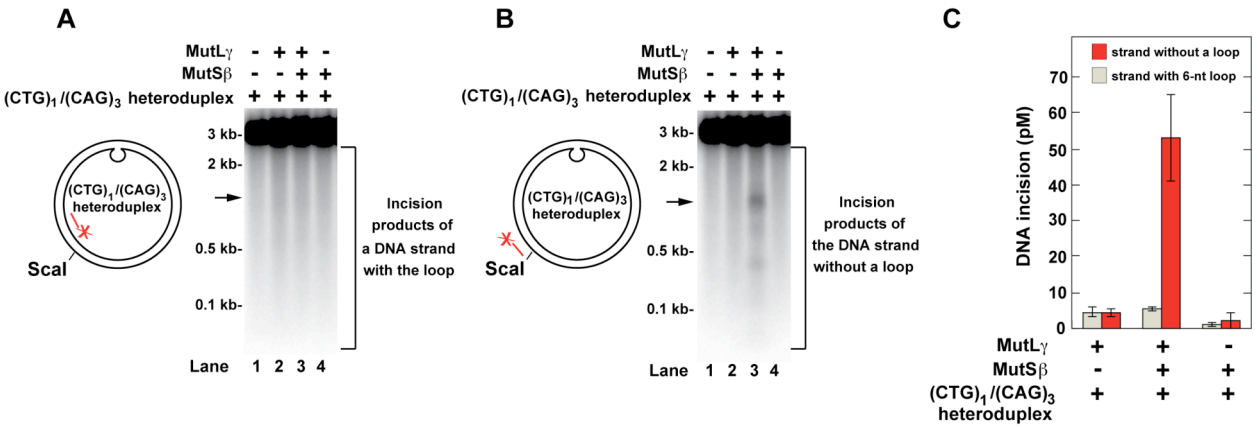

Figure S7. Cleavage of a relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA by activated human MutLγ endonuclease. Reactions were carried out and analyzed as described in **Fig. 4** except that the DNA substrate used was a relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA that contained a 6-nt loop in the bottom strand. (**A**) Activated human MutLγ does not cleave the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA in the loop-containing strand. (**B**) Activated human MutLγ incises the $(CTG)_1/(CAG)_3$ heteroduplex in the loop-lacking strand. The arrows indicate locations of DNA products that were formed by cleavage of the ccDNA at the loop site. The diagrams outline the relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA and show the relative positions of the 6-nt loop, the ScaI site, and the ^{32}P-labeled probes. (**C**) Quantification of incision of the two strands of the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA by activated MutLγ endonuclease. The data are presented as averages ± 1 S.D. ($n \geq 2$).

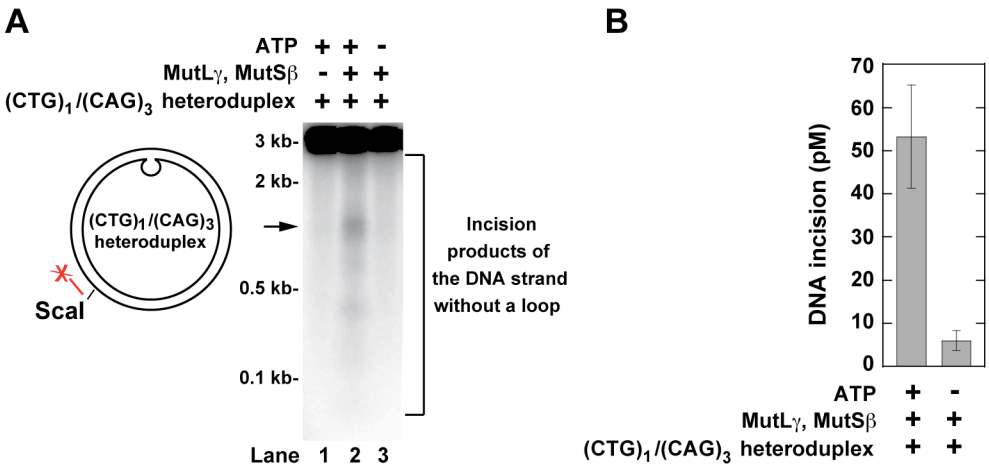

Figure S8. Human MutLγ endonuclease cleaves a relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA in an ATP-dependent reaction. Reactions were performed as described in **Fig. S7** except that one of the reactions was carried out in the absence of ATP. (**A**) ATP is required for for cleavage of the relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA by human MutLγ. Reaction products of indicated reactions were cleaved with ScaI, separated in 1.2% denaturing agarose gels, and visualized by Southern hybridizations with ^{32}P-labeled probe 5′-CAAGAGCAATCGGTCGCCGCATACAC -3′. The arrow marks location of DNA products that were formed by cleavage of the heterodplex ccDNA near the loop site. The diagram outlines the relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA and shows the relative positions of the 6-nt loop, the ScaI site, and the ^{32}P-labeled probe. (**B**) Quantification of ATP dependence of cleavage of the loop-lacking strand of the relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA by human MutLγ endonuclease. The incision values are presented as averages ± 1 S.D. ($n \geq 2$).

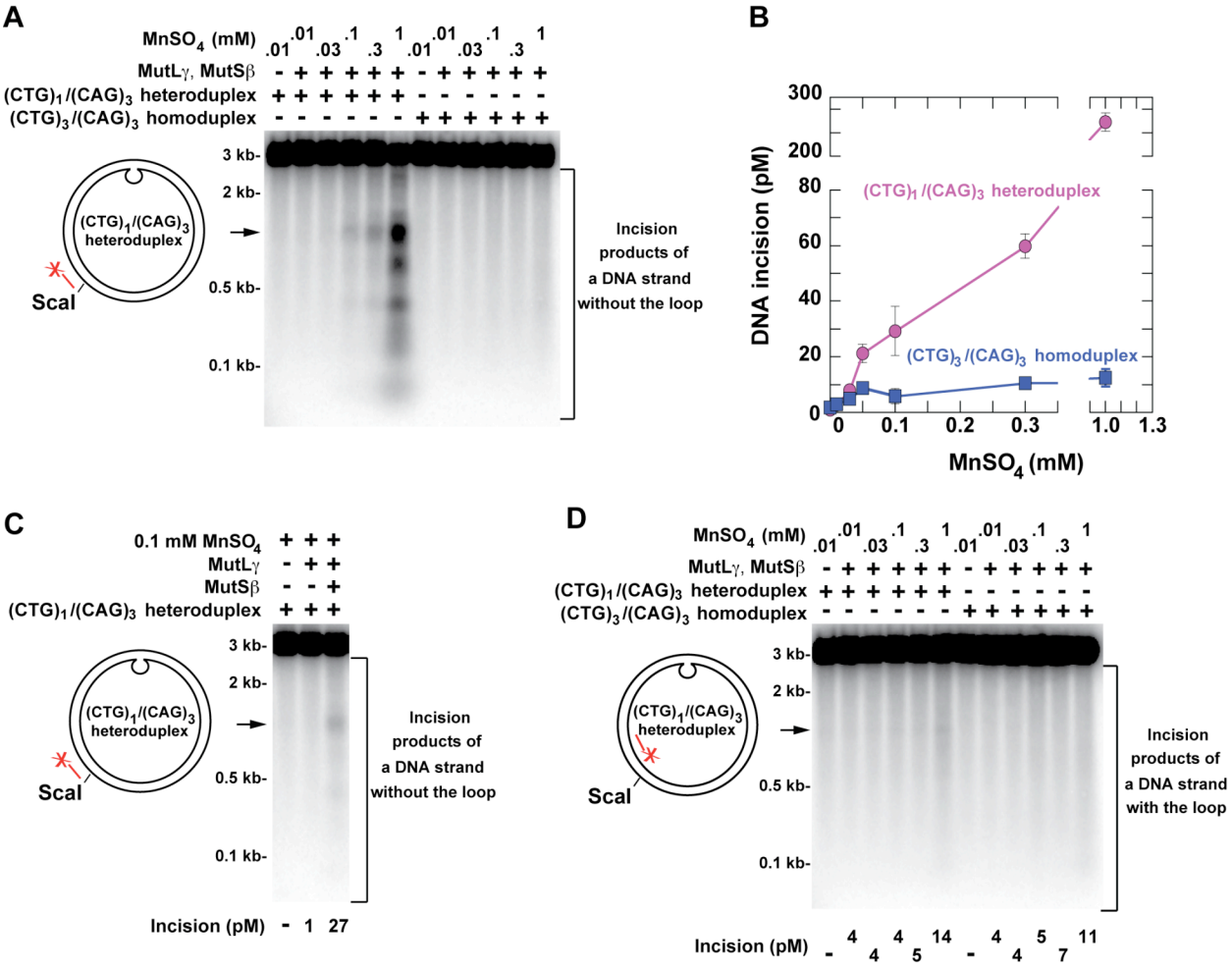

Figure S9. MutSβ-dependent endonuclease activity of human MutLγ is activated by Mn^{2+} to incise the loop-lacking strand of a relaxed $(CTG)_1/(CAG)_3$ heteroduplex ccDNA. Reactions were performed in the presence of indicated proteins and relaxed ccDNAs as described in **Fig. S7** except that the reaction mixtures lacked Mg^{2+} and included the indicated concentrations of Mn^{2+}. When present in the reaction mixtures, MutLγ and MutSβ were at 39 and 25 nM, respectively. The reaction products were cleaved with ScaI, separated on 1.2% denaturing agarose gels, and analyzed by Southern hybridizations with a ^{32}P-labeled oligonucleotide, 5′- CAAGAGCAATCGGTCGCCGCATACAC -3′ (**A-C**), that is complementary to the top strand, and with a ^{32}P-labeled oligonucleotide 5′-

GTGTATGCGGCGACCGAGTTGCTCTTG-3′ (**D**), that is complementary to the bottom strand. The arrows indicate products that were formed by cleavage of the ccDNA at the loop site. The diagrams outline the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA and show the relative positions of the 6-nt loop, the ScaI site, and the ^{32}P-labeled probe. (**A**) Cleavage of the $(CTG)_1/(CAG)_3$ heteroduplex and control $(CTG)_3/(CAG)_3$ homoduplex ccDNAs by MutLγ endonuclease in the presence of MutSβ and different concentrations of Mn^{2+}. (**B**) Incision of the loop-lacking strand of the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA as a function of Mn^{2+} concentration. The incision values are presented as averages ± 1 S.D. ($n \geq 2$). (**C**) Mn^{2+}-dependent incision of the loop-lacking strand of the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA by MutLγ endonuclease requires MutSβ. Reactions were performed in the presence of 0.1 mM Mn^{2+} as described in **A**. The incision values that are presented as averages ($n = 2$) were obtained by quantification of images including the one shown. (**D**) MutSβ-dependent endonuclease activity of human MutLγ is silent on the loop-containing strand of the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA in the presence of Mn^{2+}. The arrow indicates products that were formed by cleavage of the ccDNA at the loop site. The diagram on the left shows the $(CTG)_1/(CAG)_3$ heteroduplex ccDNA, the relative positions of the 6-nt loop, the ScaI site, and the ^{32}P-labeled probe. The incision values are presented as averages ($n \geq 2$).

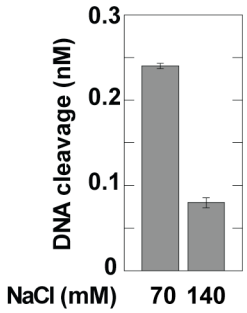

Figure S10. Mn^{2+}-dependent endonuclease activity of human MutLγ on a supercoiled DNA in the presence of different NaCl concentrations. The Mn^{2+}-dependent endonuclease reactions were performed and analyzed as described in **Fig. 1A**. The reaction mixtures contained MutLγ (22 nM) and 70 mM or 140 mM NaCl. The data shown are averages ± 1 S.D. (n = 2).

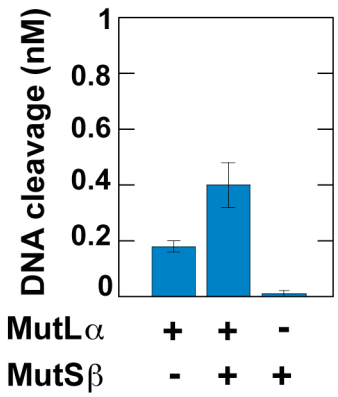

Figure S11. MutSβ stimulates the Mn^{2+}-dependent endonuclease activity of human MutLα. The Mn^{2+}-dependent endonuclease reactions were performed and analyzed as described in **Fig. 1A**. When indicated the reaction mixture contained MutLα (60 nM) and MutSβ (13 nM). The data shown are averages ± 1 S.D. (n = 4).