Supplementary material

for

Excision of the doubly methylated base N^4,5-dimethylcytosine from DNA by *Escherichia coli* Nei and Fpg proteins

by

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Repair enzyme details

Nei (Cat. No. M0299S; 10 000 units/ml, 2.1 μM); Fpg [Cat. No. M0240S; 8000 units/ml (13 μM); lot No. 0061405; dissolved in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 200 μg/ml BSA, 50% glycerol], Nth [Cat. No. M0268S; 10 000 units/ml (0.7 μM); lot No. 0031402; dissolved in 10 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 200 μg/ml BSA, 50% glycerol], Ung [Cat. No. M0280S; 5000 units/ml (1.95 μM)], hSMUG1 [Cat. No. M0336S; 5000 units/ml (0.33 μM); for assays using dsDNA, lot No. 0011512; for assays using ssDNA, lot No. 0011408; dissolved in 10 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 200 μg/ml BSA, 50% glycerol, 0.15% Triton® X-100], Nfo [Cat. No. M0304S; 10 000 units/ml (83 nM)] and PseT [Cat. No. M0201S; 10 000 units/ml (0.29 μM)] were obtained from New England Biolabs. Mug (Cat. No. 4125-100-EB; 100 units/ml; dissolved in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2.5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50% glycerol) was obtained from Trevigen. hUNG (hUNGΔ84 with His-tag; 820 μM; dissolved in 20 mM Tris-HCl, pH 7.5, 60 mM NaCl, 1 mM EDTA, 1 mM DTT) was a gift from Bodil Kavli and Geir Slupphaug. hOGG1 (with His-tag; 5.4 μM dissolved in 10 mM 4-morpholineethanesulfonic acid (MES), pH 6.0, 50 mM NaCl and 10 mM 2-mercaptoethanol) was purified as described [26]. hTDG (with His-tag; 15 μM; dissolved in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM 2-mercaptoethanol, 10% glycerol) was a gift from David Schürmann and Primo Schär. hNEIL1 [full length with His tag; 4.5 μM; dissolved in 10 mM Tris-HCl, pH 7.0, 50 mM NaCl, 10 mM 2-mercaptoethanol, 20% (v/v) glycerol], hNEIL2 (full length with His tag; 0.2 mg/ml, 5.3 μM; dissolved in the same solution as NEIL1) and NEIL3 [amino acids 1–301 with His tag; 8.6 μM; dissolved in 10 mM MES, pH 6.0, 50 mM NaCl, 10 μM 2-mercaptoethanol] proteins were purified as described [28, 29].

Supplementary Figures

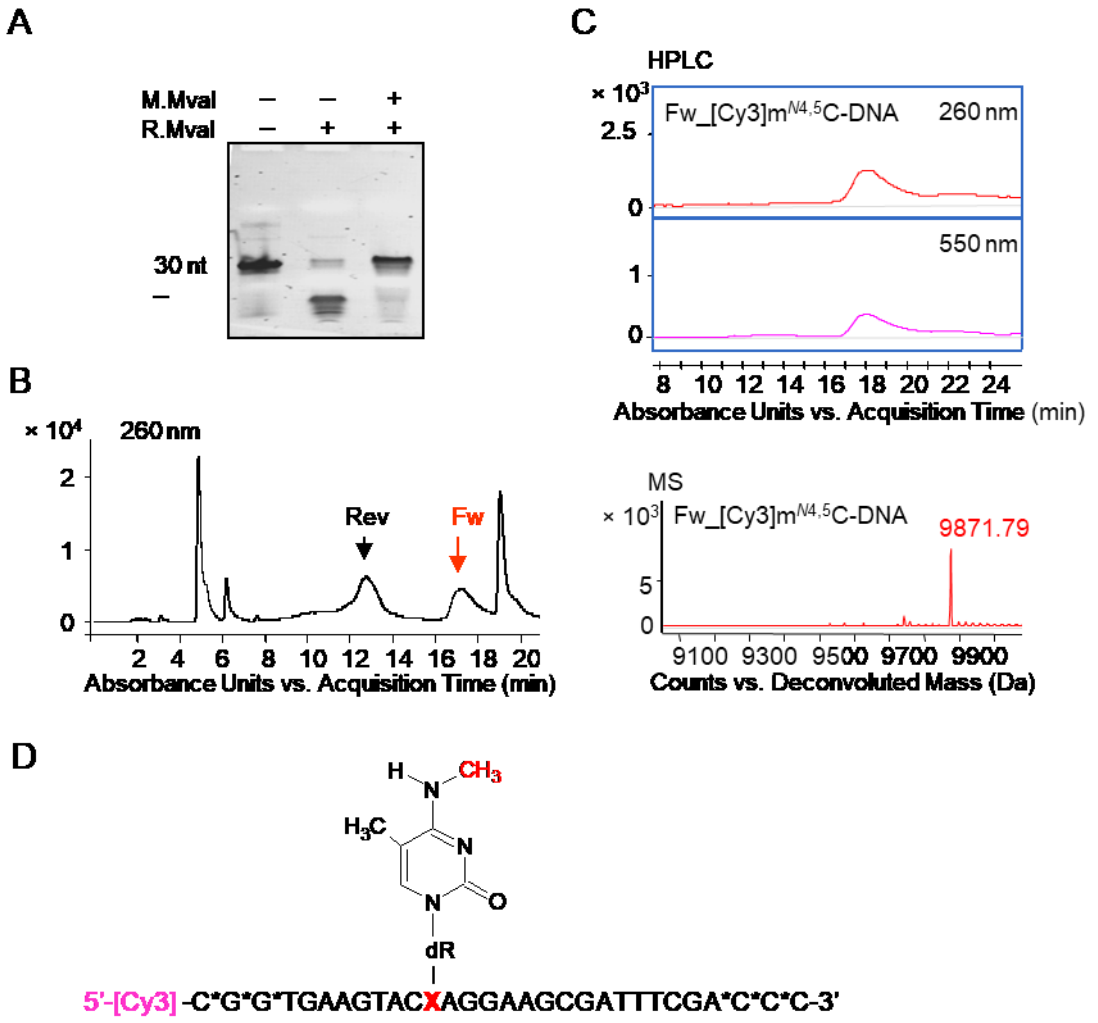

Figure S1. Preparation and characterization of $m^{N4,5}C$-DNA. (**A**) DNA protection analysis of a double-stranded 30-nt DNA containing doubly methylated cytosine residues at a unique MvaI recognition site. M.MvaI-modified (right) and unmodified control (middle) reactions were subjected to digestion by R.MvaI and analysed by denaturing 10% (w/v) PAGE. (**B**) HPLC-RP (reversed phase) purification of individual 30-nt strands from the M.MvaI reaction mixture (see A, right lane). (**C**) HPLC-MS analysis of the purified Fw oligonucleotide strand containing a Cy3 fluorophore and an internal $m^{N4,5}C$ residue. Upper panel shows the HPLC elution profile where the wavelength at 260 nm detects DNA while the signal at 550 nm results from the Cy3 fluorophore. The lower panel shows the HR-MS scan (theoretical mass 9871.72 Da). (**D**) Schematic representation of the purified oligonucleotide.

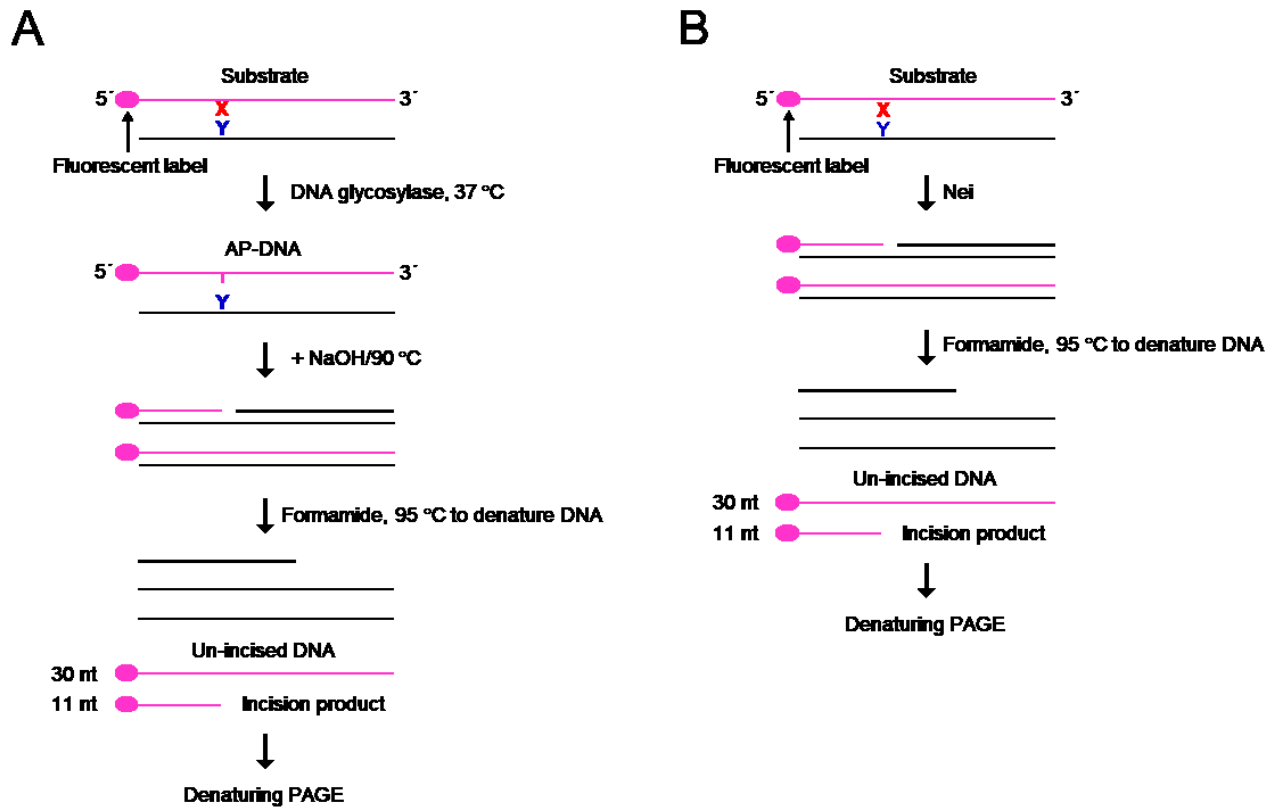

Figure S2. Schematic representation of DNA base excision and incision assays to study base excision repair (BER) enzymes. (**A**) Conventional base excision assay. (**B**) Enzyme assay employed in the current study (see 'Materials and methods').

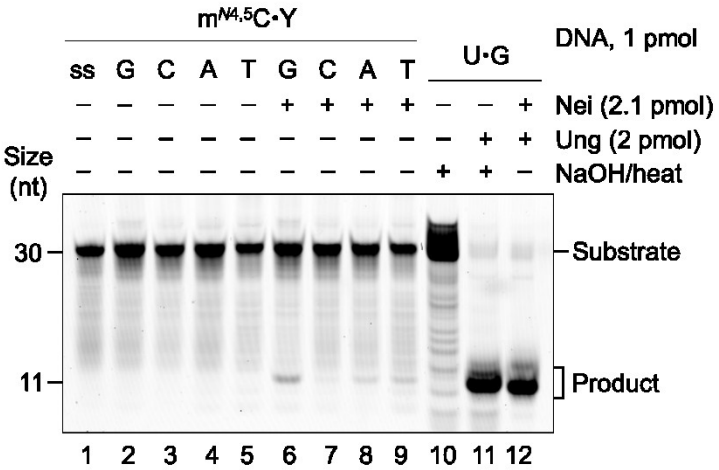

Figure S3. Incision activity of Nei at $m^{N4,5}C$ in DNA. DNA substrate (1 pmol) was incubated alone (lanes 1–5) or with Nei (2.1 pmol; lanes 6–9) at 37 °C in Nei buffer (10 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1 mM EDTA) for 1 h (final volume, 20 µl). U·G-DNA (30 nt; 1 pmol) incubated without (lane 10) and with *E. coli* Ung (lane 11) followed by NaOH/heat treatment was used as negative and positive control for active Ung, respectively, which was used to convert U·G-DNA into AP-DNA to demonstrate active Nei (*i.e.*, lyase activity; lane 12). Incised DNA was separated from un-incised DNA by denaturing PAGE at 200 V for 2 h.

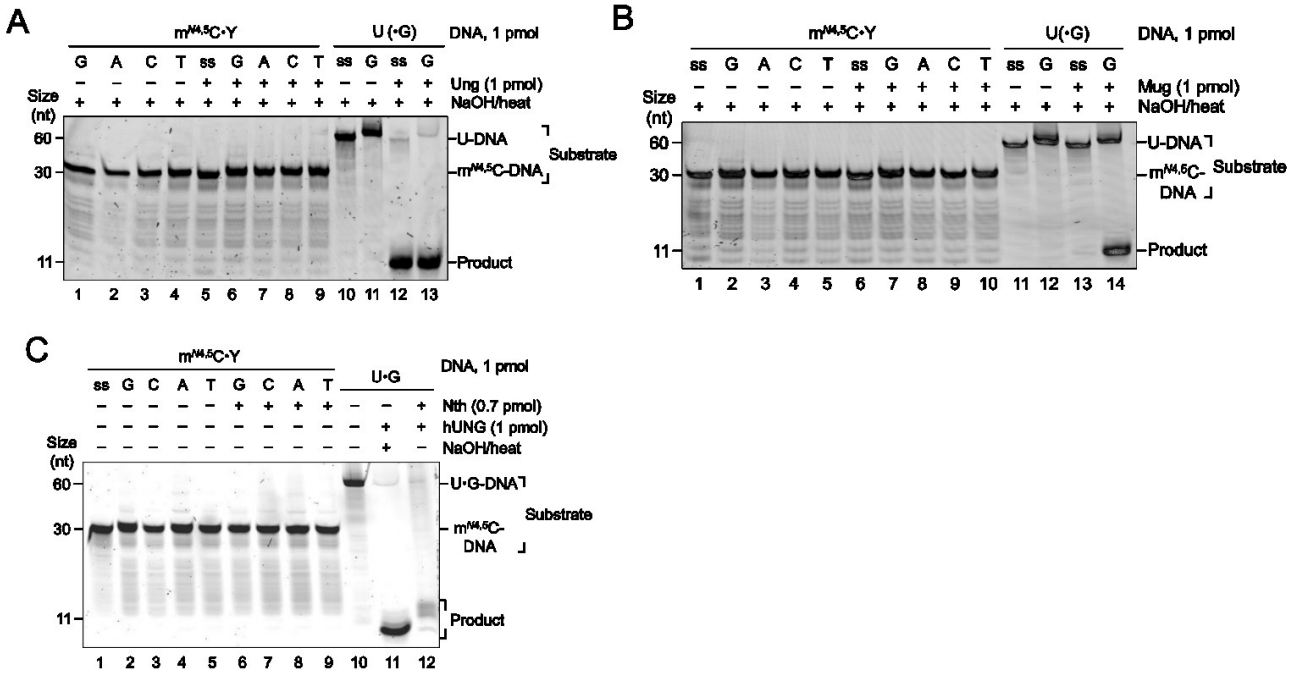

Figure S4. *E. coli* DNA glycosylases without detectable activity for $m^{N4,5}C$ in DNA. DNA substrate (Figure 1A) or U-DNA was incubated at 37 °C in the reaction buffer containing the reagents added as indicated below with or without repair enzyme for 1 h (final volume, 20 µl), if not otherwise stated. (**A**) DNA substrate (1 pmol) was incubated alone (lanes 1–4) or together with Ung (1 pmol; lanes 5–9) in 100 mM Tris-HCl, 5 mM DTT, 5 mM EDTA, pH 8.0 for 30 min. Single-stranded U- or U·G-DNA (60 nt; 1 pmol) incubated without (lanes 10–11) and with Ung (lanes 12–13) was used as negative and positive control for Ung activity, respectively. Incised was separated from un-incised DNA by denaturing PAGE (Figure S2A) at 180 V for 3 h. (**B**) DNA substrate (1 pmol) was incubated alone (lanes 1–5) or with Mug (1 pmol; lanes 6–10) in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA. Single-stranded U- or U·G-DNA (60 nt; 1 pmol) incubated without (lanes 11–12) and with Mug (lanes 13–14) was used as negative (lanes 11–13) and positive control for Mug activity (lane 14). Denaturing PAGE was performed as above at 180 V for 2 h 30 min. (**C**) DNA substrate (1 pmol) was incubated alone (lanes 1–5) or with Nth (0.7 pmol; lanes 6–9) in TE-Nth buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM DTT) containing 0.1 mg/ml BSA. U·G-DNA (60 nt; 1 pmol) incubated without (lane 10) and with hUNG (1 pmol; lane 11) was used as negative and positive control for hUNG activity, respectively, which was used to convert U·G-DNA into AP-DNA to demonstrate Nth activity (lane 12). Incubation time was 30 min. Denaturing PAGE was performed as in A at 200 V for 2 h.

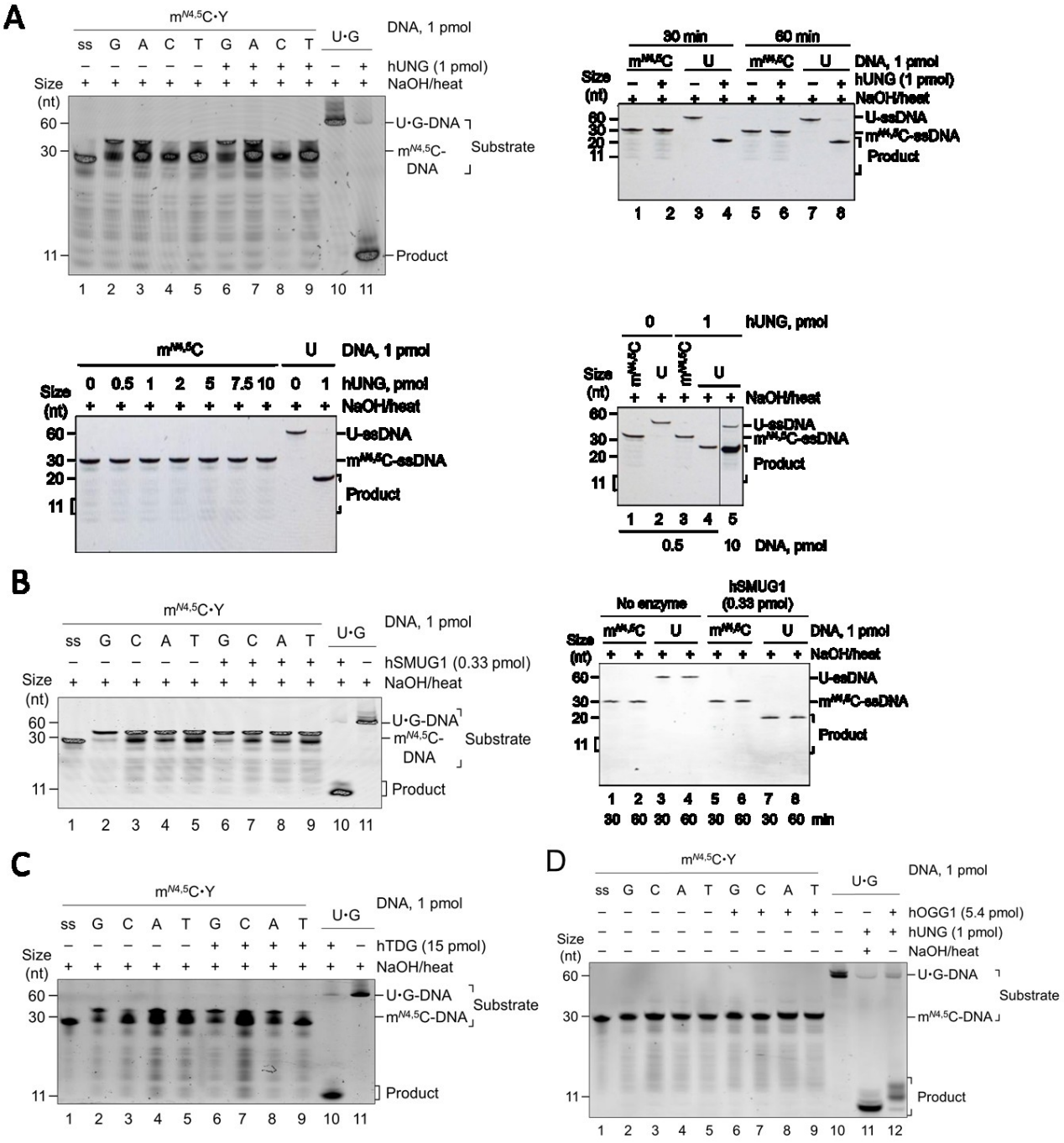

Figure S5. Human DNA glycosylases show no detectable activity for $m^{N4,5}C$ in DNA. DNA substrate (Figure 1A) or U-DNA was incubated at 37 °C in the reaction buffer with the reagents added as indicated below with or without repair enzyme and for the time periods also indicated (final volume, 20 µl). Incised was separated from un-incised DNA by denaturing PAGE (Figure S2A) at 150–180 V for 2–3 h. (**A**) Upper left panel: hUNG was incubated in 45 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.5, 0.4 mM EDTA, 1 mM DTT, 70 mM KCl, 2% glycerol, 0.1 mg/ml BSA for 30 min. The same amount of hUNG incubated with and without U·G-DNA (60 nt; 1 pmol) was used as positive and negative control for hUNG activity, respectively. Lower right panel indicates the activity of hUNG (1 pmol) at high DNA concentration. (**B**) hSMUG1 was incubated in the same buffer solution as hUNG for 1 h (left panel) or for different time periods (right panel). The denaturing PAGE was performed using a TTE (Tris-taurine-EDTA) buffer system (89 mM Tris base, 28.5 mM taurine, 0.5 mM EDTA). (**C**) hTDG was incubated in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA containing 0.5 unit of uracil-DNA glycosylase inhibitor for 1 h. The denaturing PAGE was performed as in B. (**D**) hOGG1 was incubated in NEB2 buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing 0.1 mg/ml BSA for 1 h.

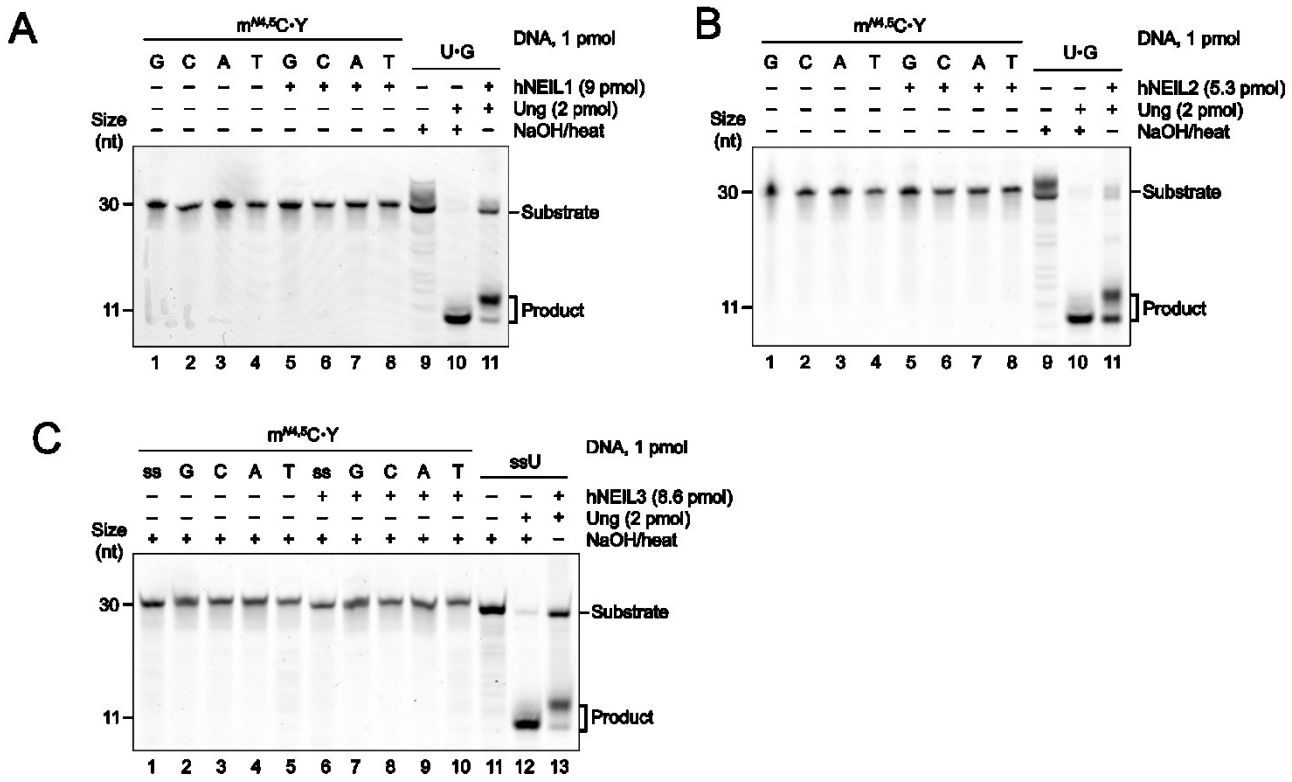

Figure S6. Human NEIL DNA glycosylases are without detectable $m^{N4,5}C$-excising or incising ability. DNA substrate (Figure 1A) or U-DNA was incubated at 37 °C for 60 min with or without repair enzyme in the reaction buffer containing the reagents added as indicated below or in the figures (final volume, 20 µl). Incised DNA was separated from un-incised DNA by denaturing PAGE (Figure S2) at 200 V for 2 h. (**A**) hNEIL1 (9 pmol) was incubated in 10 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1 mM EDTA (Nei buffer). (**B**) hNEIL2 (5.31 pmol) was incubated in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA (NEIL2 buffer) containing 0.1 mg/ml BSA. *E. coli* Ung was incubated with and without U·G-DNA (30 nt; 1 pmol) as positive and negative control for Ung activity, respectively, which was used to form AP-DNA as a positive control for active NEIL1 and NEIL2 enzymes. (**C**) hNEIL3 (8.63 pmol) was incubated in 50 mM MOPS, pH 7.5, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT (NEIL3 buffer). Ung was incubated with and without ssU-DNA (30 nt; 1 pmol) as positive and negative control for Ung activity, respectively, which was used to form ssAP-DNA as a positive control for active NEIL3 enzyme.

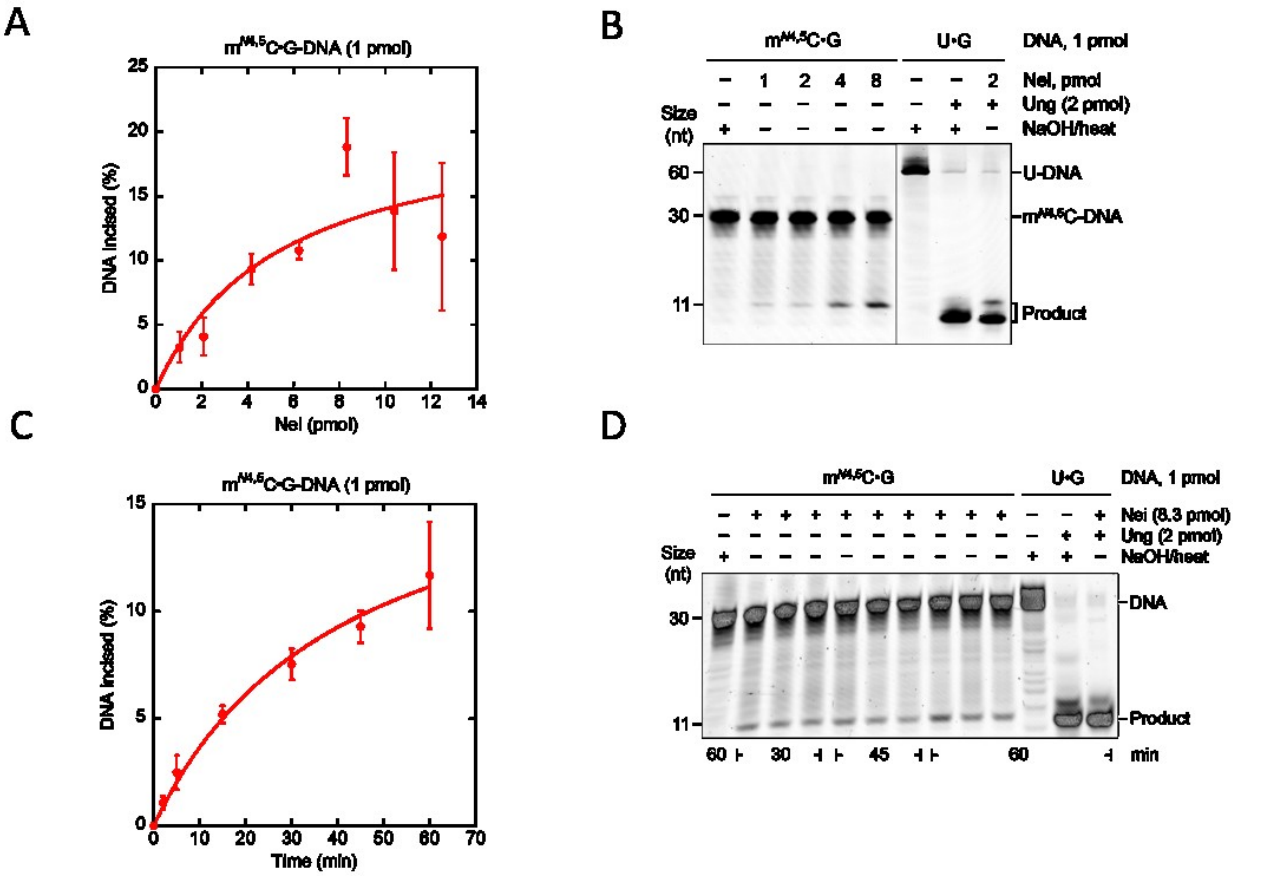

Figure S7. Nei activity for incision at $m^{N4,5}C$ opposite G in DNA as a function of time and protein concentration. (**A**) Increasing amounts of Nei up to and including 12.5 pmol were incubated with DNA substrate (Figure 1A; 1 pmol) at 37 °C (Figure S2B) for 1 h (final volume, 20 µl). Each value represents the average (± SD) of three independent experiments, where (**B**) shows parts of a typical experiment. (**C**) Nei (8.3 pmol) was incubated with DNA substrate as in A for the time periods indicated. Each value represents the average (± SD) of three independent experiments, where (**D**) shows parts of a typical experiment.

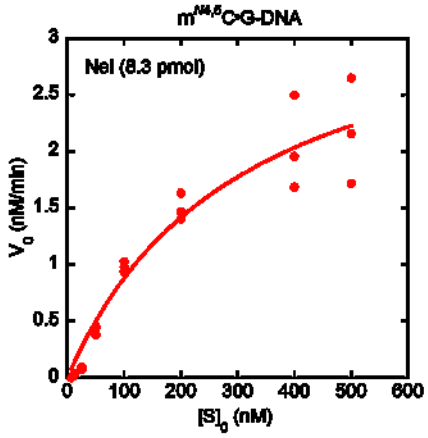

Figure S8. Nei kinetics for incision at the $m^{N4,5}C{\cdot}G$ pair in DNA. Nei (8.3 pmol) was incubated with an increasing amount of DNA substrate $[S]_0$ as indicated for 10 min using the same conditions as in Figure S7, where each of the three independently measured values of the $m^{N4,5}C$ excision rate V_0 is indicated. The graph represents the Michaelis-Menten equation $V_0 = V_{max} \times [S]_0/(K_m + [S]_0)$ (R = 0.993) from which V_{max} and K_m were determined.

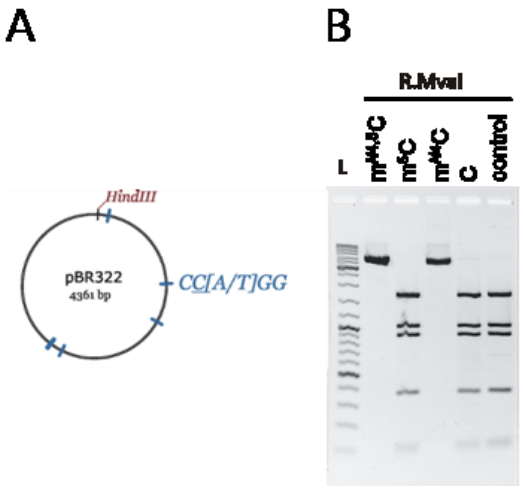

Figure S9. Preparation of plasmid DNA containing $m^{N4,5}C$. (**A**) Schematic representation of MvaI and HindIII recognition sites in pBR322 plasmid. (**B**) DNA protection analysis of M.MvaI methylation of pBR322 DNA using R.MvaI restriction endonuclease. pBR322 DNA containing double-methylated ($m^{N4,5}C$), mono-methylated ($m^{N4}C$ or $m^{5}C$) or unmethylated cytosines (C) at C<u>C</u>(A/T)GG sites (see 'Materials and methods') was digested with R.MvaI. Control, commercial pBR322 digested with R.MvaI; L, GeneRuler DNA Ladder Mix (Thermo Fisher Scientific).