Supporting Information

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SI Text

Materials and Methods. ***Glycosylases.*** Control glycosylases, including *E. coli* endonuclease III (EcoNth), *E. coli* formamidopyrimidine DNA glycosylase (EcoFpg), and *E. coli* endonuclease VIII (EcoNei), Mimivirus endonuclease VIII-2 (MvNei2), human endonuclease VIII-like 1 (NEIL1) and human endonuclease VIII-like 2 (NEIL2) were from our laboratory stocks and were purified as previously described (1–5).

DNA substrates. ^{32}P-labeled single-stranded, double-stranded, fork, or bubble structure substrates and substrates containing an AP site were prepared using standard protocols (6, 7). 30-mer or 24-mer oligodeoxynucleotides carrying Sp, Gh, and Me-FapyG were synthesized as previously described (8, 9). Note that Sp1- and Sp2-containing oligomers are designated on the basis of their elution order on a Dionex DNAPac PA-100 ion exchange column, and this order is thought to be opposite from that of the diastereomeric nucleosides studied by reversed-phase HPLC (10). 35-mer or 51-mer oligodeoxynucleotides carrying other base lesions and all complementary strands were purchased from Midland Certified Reagent Co. (Midland, TX). The sequences of oligodeoxynucleotides used are listed in Table S3.

Buffer conditions for glycosylase/Lyase activity assays. EcoFpg, EcoNei, NEIL1, and MvNei2: 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA and 100 μg/mL BSA; NEIL2: 20 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM EDTA and 100 μg/mL BSA; EcoNth: 20 mM Hepes-KOH pH 7.4, 100 mM KCl, 1 mM EDTA and 100 μg/mL BSA; MmuNeil3 wt and MmuNeil3Δ324: 20 mM Hepes-NaOH pH7.0, 50 mM NaCl, 0.01% TritonX-100, 1 mM DTT and 100 μg/mL BSA.

Normalization of GC/MS data. As shown in Table S2, the quantity of each product released by each enzyme, S_{ep}, was calculated using

$$S_{ep} \equiv \frac{1}{|R_{ep}|}\sum_{r\in R_{ep}} Q_{epr} - \frac{1}{|R_{0p}|}\sum_{r\in R_{0p}} Q_{0pr},$$

where e indexes enzymes, p indexes products, Q_{epr} is a GC/MS peak intensity, r indexes replicates, R_{ep} is a set of replicates ($e = 0$ indicates no enzyme), and $|R_{ep}|$ is the number of replicates. We report S_{ep} along with its sample standard deviation, obtained from triplicate reactions performed on the same day with the same enzyme and substrate preparations. Data shown in Fig. 4 were normalized based on the equations:

$$\hat{S}_{ep} \equiv \frac{S_{ep}}{\|S_e\|},$$

Where

$$\|S_e\| \equiv \sqrt{\sum_p S_{ep}^2}.$$

In our assay, each glycosylase was incubated with γ-irradiated calf thymus DNA containing multiple lesions in a reaction mixture where substrate concentration greatly exceeded enzyme concentration and the reaction was on the linear portion of the curve. Comparisons were made between enzymes having equal concentrations of active protein. Under the Michaelis-Menten model,

$$\hat{S}_{ep} \approx \frac{(k_{\text{cat}}^{ep}/K_m^{ep})f_p}{\sqrt{\sum_{p'}((k_{\text{cat}}^{ep'}/K_m^{ep'})f_{p'})^2}},$$

where f_p is the fraction of oxidized bases that appear as product p. Therefore, contingent upon constancy of f_p, $\hat{S}_{ep}$ serves as a single vector measure of substrate specificity (k_{cat}/K_m).

Kinetic analyses. The catalytic turnover rate $k_{\text{obs}}(\text{min}^{-1})$ was measured under single-turnover conditions. Active enzyme concentrations higher than the dissociation constant (K_d) and 2.5 nM single or double-stranded oligodeoxynucleotides containing either Sp1 or an AP site were used. At appropriate time points, reactions with Sp1 containing substrates were quenched by adding an equal volume of 0.5 M NaOH and heating at 94 °C for 2 min to measure the rate of the glycosylase reaction. Reactions with AP site containing substrates were quenched by adding formamide stop buffer to measure the rate of lyase activity. Kin-Tek Rapid Quench was used when the reactions were too fast to be terminated manually.

For measurements of K_m (nM) values under steady-state conditions, 0.02 nM active MmuNeil3 MmuNeil3Δ324 was incubated with various concentrations of Sp1:C, AP:C, and ssAP substrates in MmuNeil3 glycosylase/lyase assay buffer at 37 °C. 0.002 nM active MmuNeil3Δ324 was used in reactions with single-stranded Sp1 substrate. In each case, the total reaction volume was 100 μL and aliquots of 10 μL were removed at various time points and quenched as described above. The reaction products were separated on a 12% (w/v) polyacrylamide sequencing gel and quantitated with an isotope imaging system (Molecular Imaging System, BioRad). Initial velocities were calculated using the linear part of the reaction and kinetic parameters were calculated using GraphPad Prism 5 (GraphPad Software, Inc.).

Protein N-terminal sequencing. To determine the residue that forms a Schiff base, 200 μM MmuNeil3Δ324 or MmuNeil3Δ324 V2P was trapped with an equimolar amount of unlabeled single-stranded AP substrate in 20 μL reactions. The trapped enzyme-DNA complexes were resolved on a 12% SDS-PAGE gel and then transferred to a PVDF membrane for N-terminal sequencing using Edman degradation (performed by Biomolecular Resource Facility, The University of Texas Medical Branch, Galveston, TX).

Construction of experimental strains for determination of spontaneous mutation frequencies in E. coli. *E. coli* BW35 (KL16 wild type) strain was generously provided by Dr. Bernard Weiss, Department of Pathology, Emory University. KL16 *fpg nei mutY* triple mutant strain (amp^R, cm^R, and tet^R) was created in our laboratory as previously described by Blaisdell, et al. (11). In order to express genes under the T7 promoter, we constructed a KL16 *fpg nei mutY* (DE3) strain based on the KL16 *fpg nei mutY* triple mutant strain using the λDE3 lysogenization kit (#69734-3, Novagen) following the manufacturer's protocol. λDE3 lysogens were verified by Western blot analysis using T7 RNA polymerase monoclonal antibody (Novagen). EcoNei and NEIL1Δ56 genes were cloned into the pET30a (kan^R) vector, and MmuNeil3 Δ324 was in a modified pET-DUET vector carrying the spectinomycin resistance gene and the *E. coli* methionine aminopeptidase (EcoMAP) gene. The same modified pET-DUET vector with

spe^R and EcoMAP was used as the empty vector control. Plasmid vectors were transformed into KL16 *fpg nei mutY* (DE3) cells by electroporation using the BioRad Gene Pulser™ electroporation device (BioRad). The resulting single colonies were tested for the expression of EcoNei, NEIL1Δ56, MmuNeil3Δ324, or EcMAP using SDS-PAGE gels after 0.5 mM IPTG induction at 37 °C for 4 h, and the *fpg, nei* and *mutY* loci were checked by PCR. Cultures were kept under appropriate antibiotic selection and stored at −80 °C.

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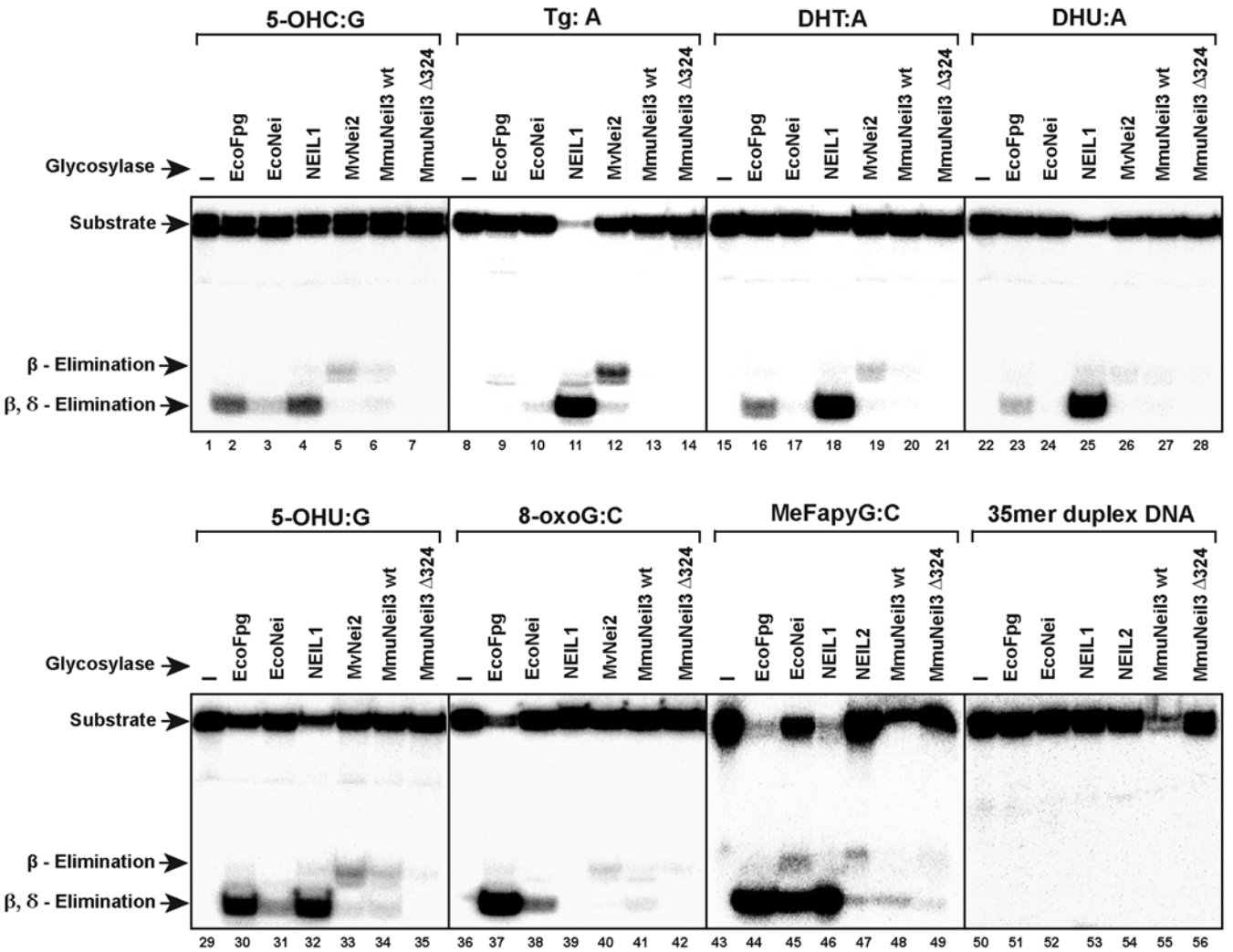

Fig. S1. DNA glycosylase/lyase assay of MmuNeil3 on damage-containing double-stranded oligodeoxynucleotides. 35 mer double-stranded substrates containing 5-OHC, Tg, DHT, DHU, 5-OHU, 8-oxoG, or a normal base G (25 nM) were incubated with 25 nM active MmuNeil3 wt (lanes 6, 13, 20, 27, 34, 41, 48, and 55), MmuNeil3Δ324 (lanes 7, 14, 21, 28, 35, 42, 49, and 56), EcoFpg (lanes 2, 9, 16, 23, 30, 37, 44, and 51), EcoNei (lanes 3, 10, 17, 24, 31, 38, 45, and 52), NEIL1 (lanes 4, 11, 18, 25, 32, 39, 46, and 53) and MvNei2 (lanes 5, 12, 19, 26, 33, 40, 47, and 54) at 37 °C for 30 min. Reactions were stopped by formamide stop buffer to show glycosylase and lyase activities. Lanes 1, 8, 15, 22, 29, 36, 43, and 50 are no enzyme controls.

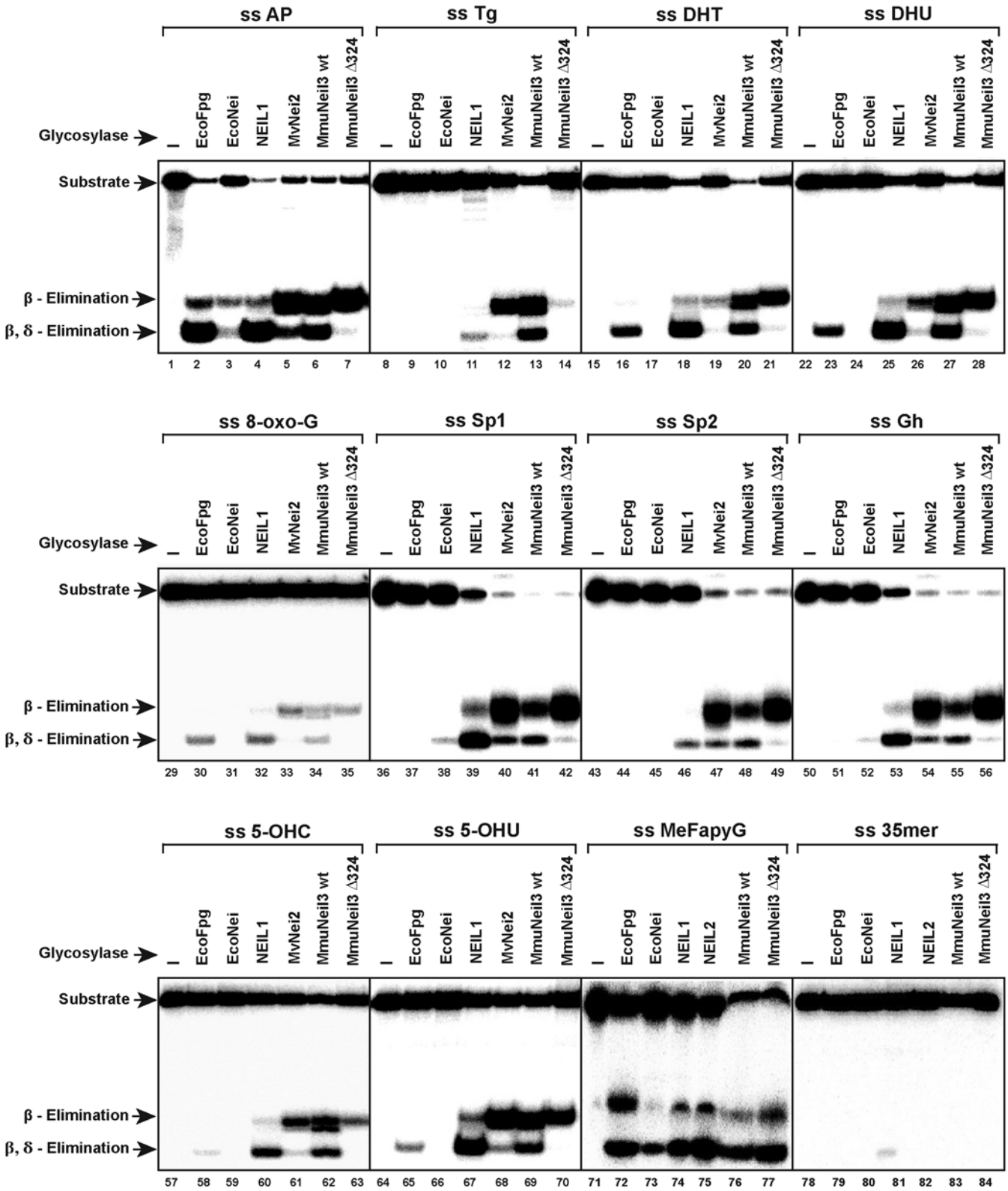

Fig. S2. DNA glycosylase/lyase assay of MmuNeil3 on damage-containing single-stranded oligodeoxynucleotides. Single-stranded substrates containing an AP site, Tg, DHT, DHU, 8-oxoG, Sp1, Sp2, Gh, 5-OHC, 5-OHU, MeFapyG, or a normal base G (25 nM) were incubated with 25 nM active MmuNeil3 wt (lanes 6, 13, 20, 27, 34, 41, 48, 55, 62, 69, 76, and 83), MmuNeil3Δ324 (lanes 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84), EcoFpg (lanes 2, 9, 16, 23, 30, 37, 44, 51, 58, 65, 72, and 79), EcoNei (lanes 3, 10, 17, 24, 31, 38, 45, 52, 59, 66, 73, and 80), NEIL1 (lanes 4, 11, 18, 25, 32, 39, 46, 53, 60, 67, 74, and 81), MvNei2 (lanes 5, 12, 19, 26, 33, 40, 47, 54, 61, 68, 75, and 82) at 37 °C for 30 min. Reactions were stopped by formamide stop buffer to show glycosylase and lyase activities. Lanes 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, and 78 are no enzyme controls.

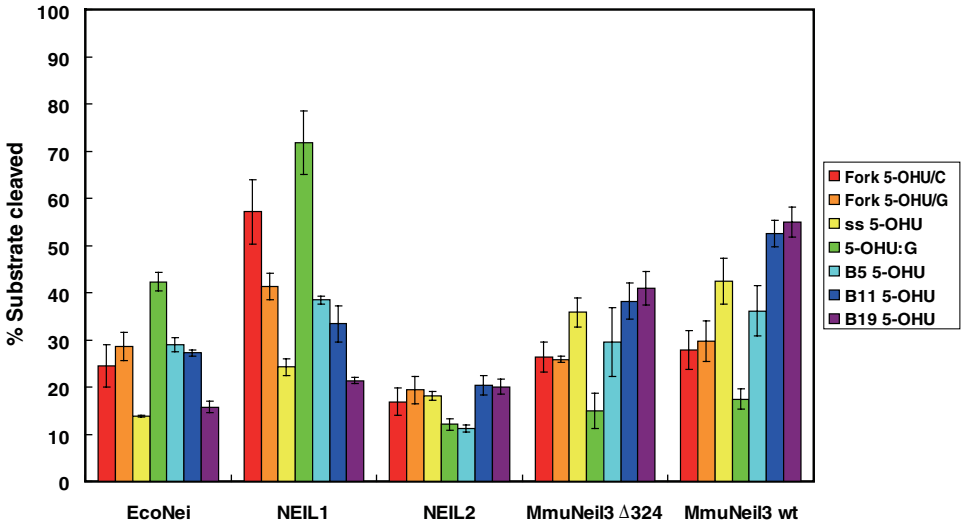

Fig. S3. Quantification of the activity of EcoNei, NEIL1, NEIL2, MmuNeil3 wt, and MmuNeil3Δ324 on substrates in different DNA structures. 25 nM enzyme was incubated with 25 nM substrate containing 5-OHU in a fork structure (Fork 5-OHU/C and Fork 5-OHU/G), single-stranded oligodeoxynucleotide, double-stranded oligodeoxynucleotide, and bubble structures, 5 nt (B5), 11 nt (B11), and 19 nt (B19) as listed at 37 °C for 30 min. Reactions were stopped by formamide stop buffer to measure glycosylase plus lyase activities. Data are expressed as means of three independent measurements. Uncertainties are standard deviations.

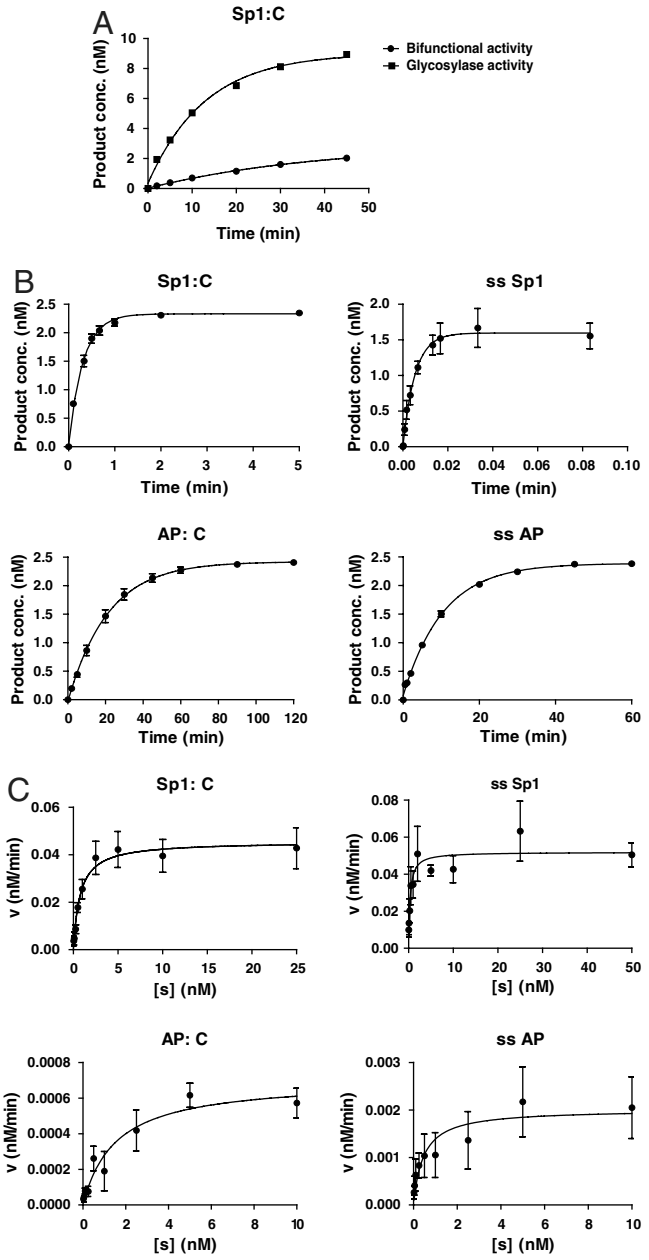

Fig. S4. Kinetics experiments with MmuNeil3Δ324. (*A*) Time course of MmuNeil3Δ324 excising double-stranded Sp1 under multiple turnover conditions. Each reaction was prepared as a pool of 100 μL mixture containing 4 nM enzyme and 10 nM double-stranded Sp1 substrate. At each time point, 10 μL aliquots were removed from the reaction mixture and quenched either by adding 10 μL 0.5 M NaOH and heating at 94 °C for 2 min (for measurements of glycosylase activity only) or by adding 10μformamide stop buffer (for measurements of the bifunctional activity). A representative experiment is shown. (*B*) Single-turnover kinetics experiment of MmuNeil3Δ324 on Sp1:C, ss Sp1, AP:C, and ss AP. Data are expressed as means of three independent measurements and fit to one phase associate model for the calculation of the catalytic turnover rate k_{obs} (min^{-1}). Uncertainties are standard deviations. (*C*) Steady-state Michaelis-Menten analysis of MmuNeil3Δ324 on Sp1:C, ss Sp1, AP:C, and ss AP. Initial velocities were calculated using the linear part of the reaction and fit to a rectangular hyperbola to derive the Michaelis constants (*K*m). Three independent measurements were shown and uncertainties are standard deviations.

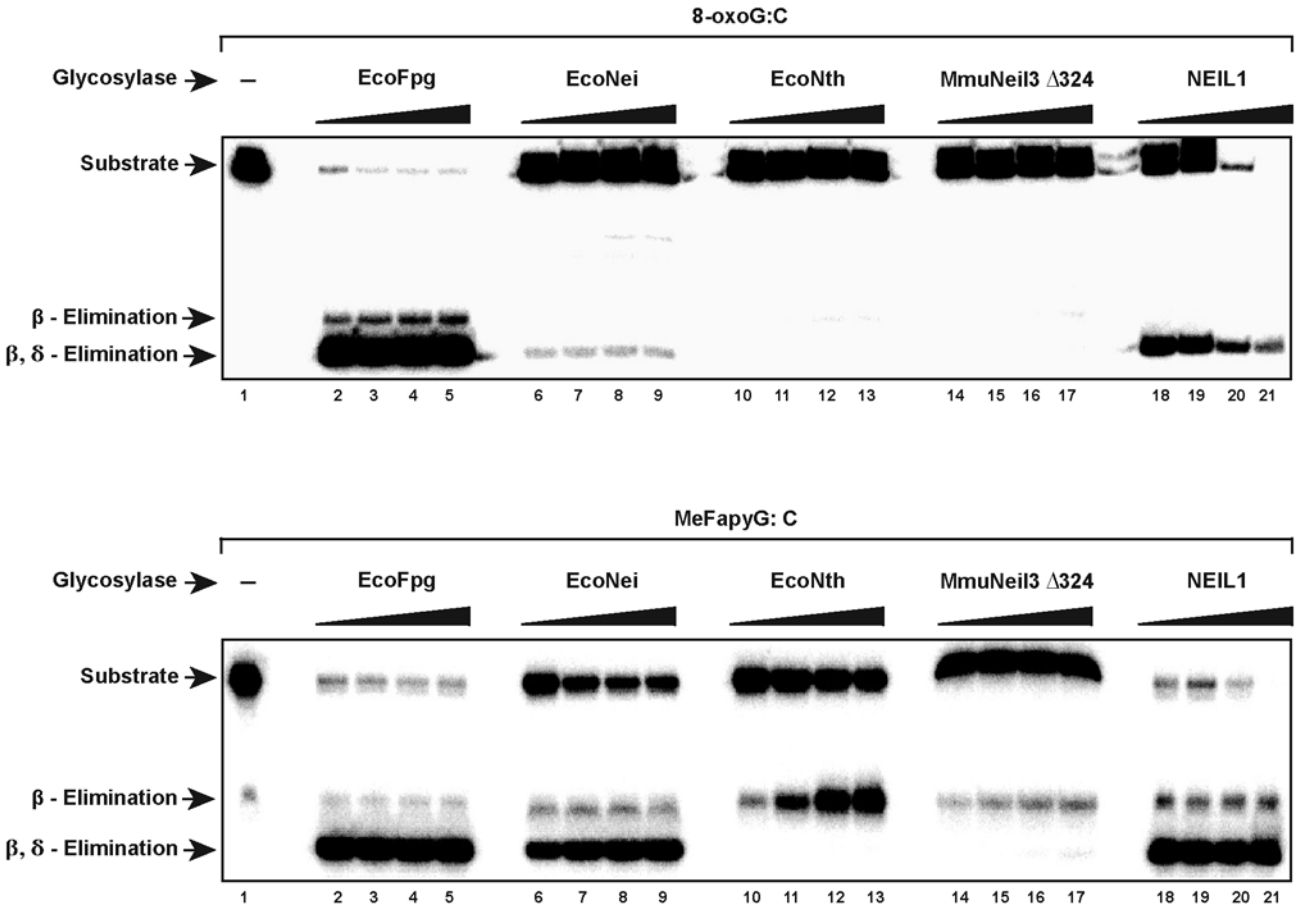

Fig. S5. DNA glycosylase/lyase assay of EcoFpg, EcoNei, EcoNth, MmuNeil3Δ324, and NEIL1 on 8-oxoG:C and MeFapyG:C under single-turnover conditions. 2.5 nM (lanes 2, 6, 10, 14, and 18), 25 nM (lanes 3, 7, 11, 15, and 19), 125 nM (lanes 4, 8, 12, 16, and 20) and 250 nM (lanes 5, 9, 13, 17, and 21) enzyme was incubated with 2.5 nM double-stranded substrate containing 8-oxoG or MeFapyG at 37 °C for 1 h. Reactions were stopped by formamide stop buffer to measure glycosylase plus lyase activities. Lane 1, no enzyme control.

Table S1. N-terminal sequences of MmuNeil3 proteins and $NaCNBH_3$ trapped protein-DNA complexes as determined by Edman Degradation.

Protein construct	Sequence	% sequence present
MmuNeil3 wt	MVEGP and VEGPG	53.6% and 46.4%
MmuNeil3Δ324	MVEGP and VEGPG	42.1% and 57.9%
MmuNeil3Δ324+ DNA	-VEGP and -EGPG	58.5% and 41.5%
MmuNeil3Δ324 V2P	PEGPG	100%
MmuNeil3Δ324 V2P+ DNA	N-terminus blocked	No sequence present

-: no amino acids detected by HPLC, cycle appeared to be blank

Table S2. DNA glycosylase activity on γ-irradiated calf thymus DNA. The numbers represent released damaged bases per 10^6 total bases from γ-irradiated DNA as measured by GC/MS.

	5OHMH	5-OHU	5-OHC	Tg	FapyA	8-oxo-A	FapyG	8-oxo-G
EcoFpg	2.1	0	0	0	212.0	23.8	731.4	634.4
EcoNei	16.8	11.0	30.7	52.0	96.9	0	0	0
EcoNth	23.2	83.1	163.5	65.1	72.1	11.8	7.0	0
NEIL1	7.9	0	0	3.4	114.1	4.5	119.1	0
MmuNeil3 Δ324	10.4	16.0	12.9	6.4	29.8	3.6	28.3	0

Table S3. DNA sequence of oligdeoxyonucleotides used as substrates.

Name	Sequence	Strand
Tg 35mer	5′-TGTCAATAGCAAG[**Tg**]GGAGAAGTCAATCGTGAGTCT-3′	U
OHU 35mer	5′-TGTCAATAGCAAG[**OHU**]GGAGAAGTCAATCGTGAGTCT-3′	U
OHC 35mer	5′-TGTCAATAGCAAG[**OHC**]GGAGAAGTCAATCGTGAGTCT-3′	U
DHU 35mer	5′-TGTCAATAGCAAG[**DHU**]GGAGAAGTCAATCGTGAGTCT-3′	U
DHT 35mer	5′-TGTCAATAGCAAG[**DHT**]GGAGAAGTCAATCGTGAGTCT-3′	U
8-oxoG 35mer	5′-TGTCAATAGCAAG[**8-oxoG**]GGAGAAGTCAATCGTGAGTCT-3′	U
U 35mer	5′-TGTCAATAGCAAG[**U**]GGAGAAGTCAATCGTGAGTCT-3′	U
35mer G	5′-TGTCAATAGCAAG[**G**]GGAGAAGTCAATCGTGAGTCT-3′	U
cG 35mer	5′-AGACTCACGATTGACTTCTCC**(G)**CTTGCTATTGACA-3′	L
cA 35mer	5′-AGACTCACGATTGACTTCTCC**(A)**CTTGCTATTGACA-3′	L
cC 35mer	5′-AGACTCACGATTGACTTCTCC**(C)**CTTGCTATTGACA-3′	L
Sp1 30mer	5′- TGTTCATCATGCGTC[**Sp1**]TCGGTATATCCCAT-3′	U
Sp2 30mer	5′- TGTTCATCATGCGTC[**Sp2**]TCGGTATATCCCAT-3′	U
Gh 30mer	5′- TGTTCATCATGCGTC[**Gh**]TCGGTATATCCCAT-3′	U
cC 30mer	5′-ATGGGATATACCGA**(C)**GACGCATGATGAACA-3′	L
MeFapyG 24mer	5′- TCATCATGCGTC**[MeFapyG]**TCGGTATATCC-3′	U
cC 24mer	5′- GGATATACCGA**(C)**GACGCATGATGA -3′	L
Fork /C 35mer	5′- AGACTCACGATTGACTTCTCC**CGAACGATAACTGT**-3′	L
Fork /G 35mer	5′- AGACTCACGATTGACTTCTCCG**GAACGATAACTGT**-3′	L
OHU 51mer	5′-GCTTAGCTTGGAATCGTATCATGTA**[OHU]**ACTCGTGTGCCGTGTAGACCGTGCC-3′	U
cG 51mer	5′-GGCACGGTCTACACGGCACACGAGT**(G)**TACATGATACGATTCCAAGCTAAGC-3′	L
B5 51mer	5′-GGCACGGTCTACACGGCACACGA**AGCCC**CATGATACGATTCCAAGCTAAGC-3′	L
B11 51mer	5′-GGCACGGTCTACACGGCACA**AACAGCCCACG**GATACGATTCCAAGCTAAGC-3′	L
B19 51mer	5′-GGCACGGTCTACACGG**ACACAACACCCCACCACCC**CGATTCCAAGCTAAGC-3′	L

U stands for the upper strand that is ^{32}P labeled; L stands for the lower strand that is complementary to the upper strand. The underlined regions in the sequence stand for the regions that are not complemented after annealing with the upper strand. Bases in [] stand for the lesions used in this study and bases in () stand for the base opposite the lesion after annealing to the upper strand.