Supporting Information

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SI Materials and Methods

Generation of Endonuclease VIII-like 3-Knockout Mice. Endonuclease VIII-like 3 (Neil3)-knockout mice were generated in collaboration with Genoway. Targeted disruption of the *Neil3* locus was achieved by replacing exons 3–5, harboring the sequence encoding the conserved DNA-binding domain H2TH, with a positive selection cassette for neomycin resistance. DNA segments located 5′ and 3′ to exons 3–5 (Table S1) were amplified by PCR using 129/SvJ genomic DNA and subcloned into vector pGT-N28 (N3728S; New England Biolabs) (Fig. S1*A*). The targeting construct was linearized by NotI and electroporated into 129/SvJ ES cells. Genomic DNA was extracted from Geneticin (G418)-resistant ESC clones, BamHI digested, and subjected to Southern blot analysis. One positive clone was identified (Fig. S1*B*), and correct 5′ and 3′ targeting was confirmed by PCR. Recombinant ES cells were injected into C57BL/6 blastocysts, which were transferred to pseudopregnant foster mothers. Chimeric male offspring were bred with C57BL/6 females to obtain *Neil3*$^{+/-}$ mice, which were backcrossed further to C57BL/6 (N8 = 99.6%). Intercrossing of F_1 heterozygous mice resulted in offspring of the expected Mendelian ratios ($^{+/+}$, 50; $^{+/-}$, 102; $^{-/-}$, 67). Genotypes were determined by PCR analysis (Fig. S1*C*), and disruption of functional Neil3 was confirmed by RT-PCR amplification of exons 1–5 using the Dynabeads mRNA DIRECT kit (610.11; Dynal) and the TITANIUM One-Step RT-PCR Kit (K1403-1; BD Biosciences) according to manufacturer's protocol (Fig. S1*D*). Experimental procedures were approved by the Norwegian Animal Research Authority.

Perinatal Hypoxia/Ischemia. Cerebral hypoxia and ischemia (HI) was produced in P9 mice by a permanent occlusion of the left common carotid artery (CCA) followed by systemic hypoxia, as previously described (1, 2), with some modifications. In brief, pups were anesthetized with isoflurane (4% induction in chamber, 2.5% maintenance on mask in a 2:1 mixture of ambient air and oxygen), and a skin incision was made in the anterior midline of the neck. The left CCA was exposed by blunt dissection and carefully separated from adjacent tissue. A needle was placed the CAA, and a monopolar cauterizer (Hyfrecator 2000; ConMed) at a power setting of 4.0 W was used to electrocoagulate the artery. The neck incision was closed with absorbable sutures (Safil 8–0 DRM6; B. Braun Melsungen AG). The surgical procedure was completed within 5 min. After a recovery period of 1–2 h, the pups were exposed to a hypoxic (10% oxygen balance nitrogen; Yara), humidified atmosphere for 60 min at 36.0 °C. The pups were returned to their dam and after 1, 3, 10, or 42 d brains were retrieved and prepared for immunohistochemistry, cell culture experiments, or quantitative RT-PCR (qRT-PCR). Sham-operated animals were subjected to anesthesia and skin incision but not to occlusion of the CCA or hypoxia.

qRT-PCR. Total RNA was isolated from subregions of the brain/neurospheres using Total RNA Kit (E.Z.N.A, Omega; Bio-Tek)/RNeasy (Qiagen), following the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in an MJ Research PTC-100 thermal cycler according to the manufacturer's protocol. qRT-PCR was performed with 50 ng/5 ng cDNA for both the target and the reference gene (GAPDH) using the SYBR Green PCR Master mix in an ABI PRISM 7300 a StepOnePlus real-time PCR System (Applied Biosystems) with the preset standard cycling conditions. Primers (Table S1) were used at a concentration of 400 nM/100 nM, and samples were run in triplicate. Quantification was done using the comparative cycle threshold (C_T) method of relative quantification (RQ), where RQ is the normalized signal level in a sample relative to the normalized signal level in the corresponding calibrator. The C_T indicates the fractional number at which the amount of amplified target reaches a fixed threshold. The C_T for the target gene was normalized to the C_T for the reference gene in the same sample, giving ΔC_T sample. The average ΔC_T from corresponding brain regions in sham-operated animals or day 2 for propagation of neurospheres were used as the ΔC_T calibrator for the calculation of $\Delta\Delta C_T$. RQ is defined as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ sample – ΔC_T calibrator.

Immunohistochemistry and Imaging. Mice were deeply anesthetized with a single dose of fentanyl/fluanisone plus midazolam s.c. and were perfused transcardially with PBS followed by cold 4% paraformaldehyde. Brains were removed and immersion fixed in 4% paraformaldehyde for at least 24 h and then were dehydrated and embedded in paraffin. To visualize both the ipsilateral and contralateral subventricular zone (SVZ), coronal sections (4 μm thick) were obtained between bregma 0.3 mm and 0.4 mm. For immunostaining, antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) before blocking with 5% goat serum (vol/vol) + 5% bovine serum albumin (wt/vol) in PBS with 0.1% Tween (vol/vol) for 30 min. 3,3′ diaminobenzidine staining (Animal Research Kit; Dako) was performed according to the manufacturer's protocol. For immunofluorescence, sections were incubated with primary antibodies at 4 °C for 16 h, followed by incubation with the appropriate fluorophore secondary antibodies for 60 min at room temperature. Nuclei were counterstained with DAPI (Sigma). Imaging was performed on a Zeiss Axiovert 200M microscope for immunofluorescence and a Zeiss Axioplan 2 microscope for bright field. All images were processed and quantified using ImageJ 1.42q software (National Institutes of Health).

The infarct area was determined by subtracting the area of undamaged, MAP2$^+$ tissue in the ipsilateral hemisphere from that of the intact contralateral hemisphere, thereby correcting for brain edema. Relative infarct area was expressed as the percentage of the contralateral hemisphere. Evenly distributed sections throughout the whole forebrain were obtained 42 d after injury to perform volume calculations of MAP2 immunoreactive tissue by Cavalieri's principle, where $V = \sum APt$ (V is the total volume, $\sum A$ is the sum of the areas measured, P is the inverse of the section sampling fraction, and t is the section thickness).

Densiometric measurements in the SVZ were performed on sections from *Neil3*$^{+/+}$ and *Neil3*$^{-/-}$ brains processed in pairs on the same microscope slides. Micrographs captured under identical settings were analyzed for fluorophore signal intensity in the SVZ, measured in a 50-μm–wide zone along the entire lateral border of the lateral ventricles.

Cell counting was performed in micrographs captured under identical settings in two predetermined regions of the striatum and one region of the SVZ per hemisphere per section.

Antibodies. The primary antibodies and dilutions used were mouse anti-MAP2, 1:8,000 (M4403; Sigma); mouse anti-nestin, 1:200 (MAB353; Millipore); rabbit anti-GFAP, 1:2,000 (18–0063; Invitrogen); mouse anti-GFAP, 1:1,500 (G3893; Sigma); rabbit anti-doublecortin, 1:2,000 (ab18723; AbCam); mouse anti-doublecortin,

1:500 (611706; BD Transduction); rabbit anti–phospho-histone H3 (serine 10), 1:800 (06–570; Upstate/Millipore); mouse anti-O4, 1:200 (MAB345; Millipore); mouse anti–gamma-H2AX, 1:500 (05–636; Upstate/Millipore); and rabbit anti-Iba1, 1:2,000 (019–19741; Wako). The secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes/Invitrogen), both at a dilution of 1:500.

Neurosphere Propagation and Differentiation. Neurospheres were propagated as previously described, with modifications (3, 4). In brief, brains were removed from *Neil3*$^{+/+}$ and *Neil3*$^{-/-}$ mice 3 d post HI and were sliced coronally to reveal the lateral ventricles, allowing isolation of the lateral SVZ and striatum from both hemispheres. In each experiment, tissue from three to six animals was pooled to achieve high cell densities in cultures. Tissue was dissociated mechanically to achieve single cells. The cell suspension then was passed through a 70-μm Nitex screen, and cell concentration and viability were measured by trypan blue dye exclusion. Cells were cultured in serum free Neurobasal-A Medium (Gibco/Invitrogen) with 2 mM L-glutamine (GlutaMAX; Gibco), 2% B-27 supplement (Gibco), 10 ng/mL human FGF-2 (MACS; Miltenyi Biotec), 20 ng/mL human EGF (R&D Systems), and penicillin/streptomycin (Pen-Strep 100×; MP Biomedicals/Fisher Scientific) at a concentration of 20,000 live cells/mL. Neurospheres grown for 7 d were trypsinized, dissociated, washed, and filtered before resuspension in medium for further propagation, exposure experiments, knockdown experiments, or differentiation. Low-passage neurospheres (P1–P2) were used throughout the study except for the knockdown experiments, in which P7–P9 neurospheres were used.

For estimates of colony forming units (CFU), 60,000 live cells/mL were seeded, and after 4 d all neurospheres >50 μm in diameter in five visual fields (5× magnification) of the T75 culture flask were counted. CFU estimates in individual flasks were calculated by dividing the sum of all spheres by the number of plated cells adjusted for area.

For differentiation experiments, cells were plated onto poly-L-lysine–coated plates at a concentration of 100,000 live cells/mL in medium lacking EGF and were incubated for 5 d. Differentiated cells were prepared for immunocytochemistry as previously described (5). Briefly, cells were washed, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS before blocking for 30 min and incubation with primary antibodies overnight. Visualization and imaging was performed as described above. Cells were counted in four visual fields (10× magnification) from three parallel wells per culture.

Knockdown of Neil3 in naïve neurospheres was achieved by using the Amaxa Mouse NSC Nucleofector Kit (VPG-1004; Lonza) in combination with the Amaxa Nucleofector II device. Neurospheres were trypsinized and filtered through a 70-μm Nitex screen at day 5 of passage 8 to obtain single cells. Approximately 1.4×10^6 cells were used for each transfection reaction. Cell pellets were resuspended in 100 μL Mouse NSC Nucleofector solution (82 mL of Nucleofector solution plus 18 mL of supplement), and 2.5 μg Neil3 shRNA plasmid or copGFP control plasmid was added (sc-61171-SH and sc-108083, respectively; Santa Cruz Biotechnology). The cell/DNA suspension was transferred to a cuvettete and placed in the Nucleofector device. Immediately after transfection, using program A-033, 0.5 mL neurobasal medium was added, and the sample was transferred to a T75 culture flask containing 19.5 mL medium. Samples were collected at 24, 48, and 72 h posttransfection for qRT-PCR analysis. For CFU estimates 72 h posttransfection, all neurospheres >20 μm in diameter in 10 random visual fields (5× magnification) of the T75 culture flask were counted. CFU estimates in individual flasks were calculated by dividing the sum of all spheres with the number of plated cells adjusted for area.

Induction of DNA Damage in Neurospheres. To induce DNA damage, neurospheres were trypsinized and passed through a 70-μm Nitex screen at day 4 of passage 8 to achieve single cells. The cell suspensions were exposed to paraquat (85617-7; Aldrich) (200 μM in neurobasal medium) for 5 h, then rinsed in PBS and cytospun onto slides using a Cytospin 4 (Thermo Scientific) at 600 rpm for 3 min or pelleted for Western blotting. Immunohistochemistry was performed as for differentiation experiments.

For Western blot analysis, cells were lysed by boiling for 5 min in 1× NuPAGE LDS Sample Buffer (NP0007; Life Technologies, Invitrogen) and subsequently sonicated for 12 s using a microtip (Vibra-cell Sonics; Sonics and Materials). Protein extracts were run on 12% polyacrylamide gels. Following gel electrophoresis, proteins were transferred to membranes using the iBlot Dry Blotting System (Life Technologies; Invitrogen). The membranes were incubated with the mouse purified monoclonal primary antibody anti-gamma-H2AX (Ser139) (05-636; Upstate/Millipore) or with the rabbit polyclonal primary antibody anti–β-Actin (ab8227; Abcam) at 4 °C overnight followed by incubation with peroxidize-labeled goat anti-mouse (115-036-068; Jackson ImmunoResearch) or goat anti-rabbit (ab6721; Abcam) secondary antibodies, respectively, for 1 h at room temperature. The membrane was treated with chemiluminescence reagent Immun-Star WesternC Chemiluminescence Kit (170-5070; Bio-Rad), and bands were visualized and quantified by the ChemiDoc XRS system (Bio-Rad).

Preparation of Hydantoin-Containing DNA Oligomers. DNA 30mers containing 8-oxo-7,8-dihydroguanine (8ohG) were synthesized by the DNA/Peptide Core Facility at the University of Utah using commercially available phosphoramidites (Glen Research) and following the manufacturer's protocols for cleavage and deprotection. HPLC-purified oligomers were oxidized under conditions previously reported to produce the hydantoins spiroiminodihydantoin (Sp) or guanidinohydantoin (Gh) from the 8ohG base in the DNA strand (6). Oligomers were purified by ion-exchange HPLC, and their identities were confirmed by electron-spray ionization-MS.

Assays for Enzymatic Cleavage of DNA. Whole-cell protein extracts were made from neurosphere cultures 4 d after first passage and from subregions of the brain 3 d post HI. Cells and tissue were lysed in buffer [50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), 200 mM KCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, 2 mM PMSF] by 30-min incubation on ice. Oligonucleotides containing specific base lesions at distinct positions (Table S2) were ^{32}P–end-labeled by T4 polynucleotide kinase (PNK) (New England Biolabs) and [γ-^{32}P]adenosine triphosphate (Perkin-Elmer) by incubation for 30 min at 37 °C. PNK was deactivated at 80 °C, and complementary oligonucleotides were annealed by heating the samples to 90 °C for 2 min followed by slow cooling to room temperature. The oligonucleotides were purified by 20% native PAGE and visualized by storage phosphor autoradiography (Typhoon 9410; Molecular Dynamics). Radiolabeled substrates were isolated from the gel, eluted in dH_2O, and stored at 4 °C. Incision assays were performed as previously reported (7) with modifications. To determine the linear range of basal incision activity in sham neurospheres, dose-dependent titration reactions were performed. Extracts then were diluted in buffer until all samples reached an equal protein concentration, and assays were performed with appropriate protein amounts for the linear range. The purified core catalytic domain of Neil3 DNA glycosylase was used as positive control (8). Ten femtomoles of DNA substrate, 4 μg or 0.4 μg (uracil assay) whole-cell extract, and 100 ng single-strand competitor DNA (35-nt random sequence) were mixed in reaction buffer [50 mM Mops (pH 7.5), 1 mM EDTA, 5% glycerol, 1 mM DTT, and ^{32}P-labeled DNA) in a total volume of 10 μL and incubated for 60 min at 37 °C. Ten microliters of formamide loading dye was added, and the samples were denatured at 80 °C

for 10 min. The reaction products were resolved in 20% denaturing polyacrylamide gels and visualized by autoradiography as done for the substrates. Incision activity was calculated using the ImageQuant v5.0 software (Molecular Dynamics). Neurospheres and tissue from two individual HI experiments were used. In each experiment, the SVZ and striatum from three to six mice exposed to HI and two or three sham-operated mice per genotype were pooled.

Statistics. GraphPad Prism version 5.01 software was used for designing graphs and for all statistical calculations. A two-tailed *t* test was applied to obtain *P* values.

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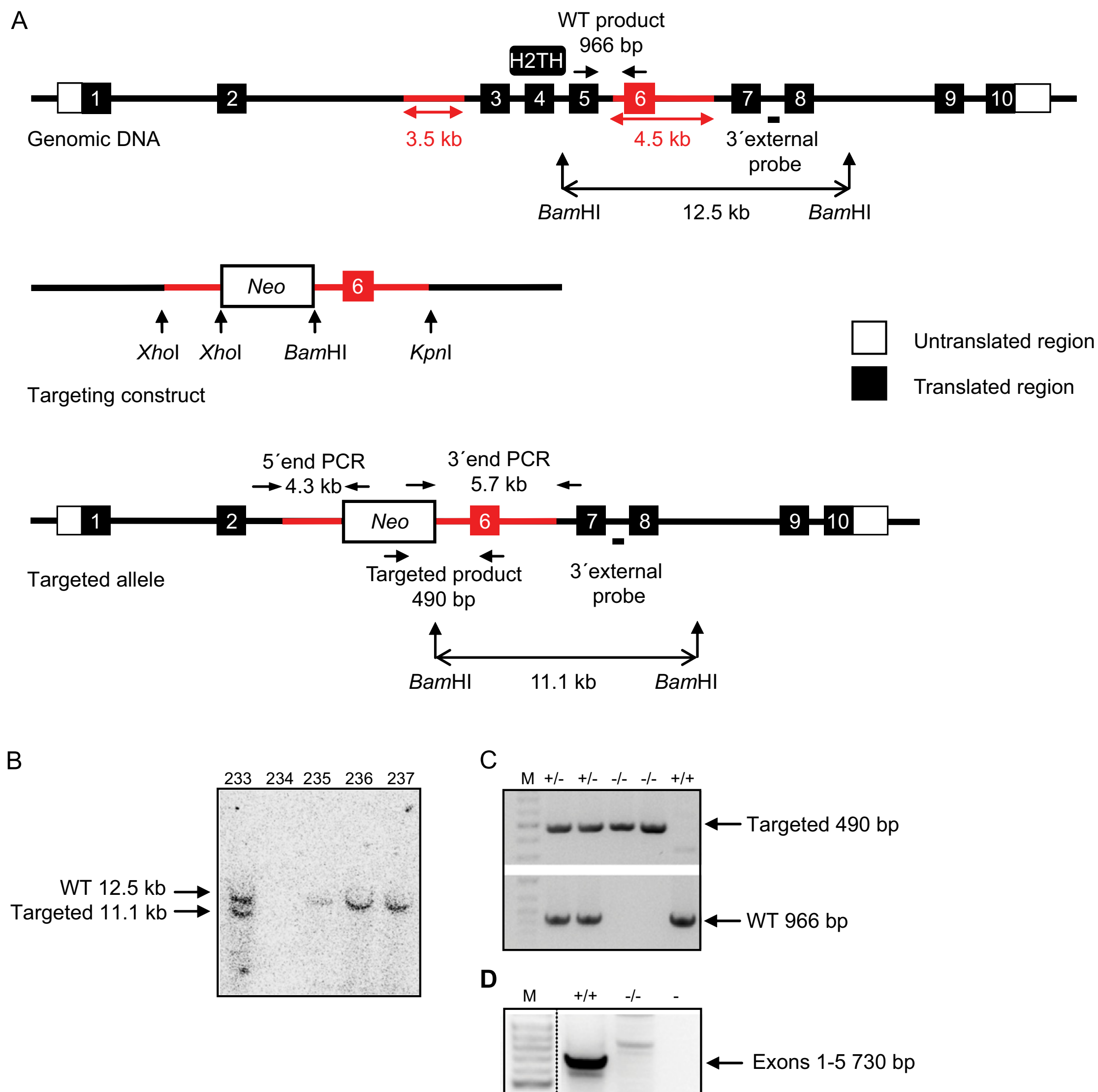

Fig. S1. Targeted disruption of the murine Neil3 locus. (*A*) Neil3-knockout mice were generated by targeted deletion of exons 3–5. Restriction sites relevant for subcloning and for Southern blot analysis, the 3′ probe used to screen ES cells for correct targeting events, and primers used to screen ES cell clones and to genotype mice are indicated. (*B*) Southern blot analysis of Geneticin (G418)-resistant ES cell clones. (*C*) Typical genotyping results obtained by PCR. (*D*) RT-PCR amplification of exons 1–5 (730 bp). M, GeneRuler DNA ladder mix (SM033; Fermentas).

Fig. S2. Perinatal HI induces late increase in expression of *Neil3* mRNA in the striatum and hippocampus. (*A*) Micrographs demonstrate the extent of brain damage in a *Neil3*$^{+/+}$ mouse after HI by H&E staining and by MAP2 immunoreactivity (brown). Whole coronal sections are displayed by merging two pictures obtained with a 2.5× objective. CTX, cortex; HC, hippocampus; STR, striatum; SVZ, subventricular zone; TH, thalamus. (Scale bar, 1 mm.) (*B*) Enlargements of regions indicated by white squares in *A*. Nuclei were counterstained with hematoxylin (blue). (Scale bar, 50 μm.) (*C*) *Neil3* expression in subregions of the ipsilateral and contralateral hemispheres 3 d after injury. Relative quantity (RQ) compared with sham-operated animals was calculated for each subregion individually. Sham values are normalized to 1.0, indicated by the gray dashed line. $^{*}P = 0.028$; $^{\#}P = 0.019$. HI, $n = 6$; sham, $n = 4$.

Fig. S3. Tissue loss in the posterior forebrain after HI. Infarction size visualized by loss of MAP2 immunoreactivity (brown) in level with the anterior hippocampus (bregma −1.6) demonstrates a profound and sustained damage to the hippocampus. *P = 0.033. n = 5–6 mice per time point. (Scale bar, 2 mm.)

Fig. S4. Neil3-deficient brains do not accumulate double-strand breaks. Nuclei are visualized with DAPI. Data are represented as mean ± SEM. *n* = 5 mice per genotype. (*A*) Double-strand breaks in DNA as shown by immunoreactivity to γH2AX (red), indicated by arrowheads. A doublecortin antibody was used to recognize neuronal progenitors (green). Cells were counted in the entire hemisphere of two nonadjacent sections from each animal. (Scale bar, 50 μm.) (*B*) Apoptosis as determined by TUNEL labeling (orange). Cell counting was performed in two nonadjacent sections from each animal. **P* = 0.016. (Scale bar, 200 μm in micrographs of the hippocampus; 50 μm in high-resolution pictures.)

Fig. S5. Cellular response to HI in the hippocampus. Densiometric quantification of nestin^{+} and GFAP^{+} cells in the subgranular zone of the hippocampus 3 d after HI. Fold change from sham-treated animals was calculated. (Scale bar, 200 μm.)

Fig. S6. DNA strand breaks (γH2AX^{+}) in dissociated naïve neurospheres after 5 h exposure to paraquat. Bar graph shows quantification of γH2AX relative to actin in Western blots.

Table S1. DNA oligonucleotides used in PCR and RT-PCR

Primer	Sequence	Product (bp)
5′ arm fwd	5′ ccggctcgagaaagacagctgcatcacca 3′	3522
5′ arm rev	5′ ccggctcgagaaacatacacgcacgcagac 3′	
3′ arm fwd	5′ cgggatccttctgcctacatcaaattgggcat 3′	4515
3′ arm rev	5′ ggggtaccactggccactactgactgacaag 3′	
3′ probe fwd	5′ cttgtcctgacacagtgtgtgc 3′	807
3′ probe rev	5′ caaaaccaaatgtgaagtttacc 3′	
5′ PCR screen fwd	5′ cag tcttcggtaagatctaagc 3′	4300
5′ PCR screen rev	5′ catagccgaatagcctctcc 3′	
3′ PCR screen fwd	5′ ctgaatgaactgcaggacgag 3′	5700
3′ PCR screen rev	5′ ctatgtgttaattctcataaacc 3′	
WT fwd	5′ caacttgcctcactctgaatgtattttgctacagt 3′	966
WT/Target rev	5′ cgaggaagctgctctctcagattc 3′	
Target fwd	5′ gcagcctctgttccacataca 3′	490
Exons 1–5 fwd	5′ gaagggccagggtgtacact 3′	730
Exons 1–5 rev	5′ gaatgctgaaatcccgagtc 3′	
Neil3 fwd RT-qPCR	5′ gggcaacatcatcaaaaatgaa 3′	
Neil3 rev RT-qPCR	5′ ctgtttgtctgatagttgacacacctt 3′	
GAPDH fwd	5′ tcgtcccgtagacaaaatggt 3′	
GAPDH rev	5′ cgcccaatacggccaaa 3′	

Table S2. Substrates used in activity assays related to experimental procedures

Lesion	Substrate oligonucleotide
Gh and Sp*	5′ tgttcatcatgcgtc[Gh/Sp]tcggtatatcccat 3′
5ohC[†]	5′ gcatgcctgcacgg[5ohC]catggccagatccccgggtaccgag 3′
5ohU[‡]	5′ gcatgcctgcacgg[5ohU]catggccagatccccgggtaccgag 3′
U*	5′ gcatgcctgcacgg[U]catggccagatccccgggtaccgag 3′

*Double-stranded substrates contained a complementary C opposite the lesion.
[†]Double-stranded substrate contained a complementary G opposite the lesion.
[‡]Double-stranded substrate contained a complementary A opposite the lesion.