Supporting Information

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SI Text

Preparation and Purification of Edited and Unedited Forms of NEIL1. An expression plasmid coding for the unedited form of NEIL1 was prepared by replacing guanine 725 of NEIL1 cDNA sequence with adenine using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and the appropriate primers (forward: 5′-CAGTTGGGGGGCAAGGGCTACGGGTCAG-3′, reverse: 5′-CTGACCCGTAGCCCTTGCCCCCCAACTG-3′, Invitrogen). Edited and unedited NEIL1 were purified from Rosetta (DE3) pLysS *Escherichia coli* strains (Novagen) using the appropriate pET30a plasmid as previously described (1). The active enzyme concentration was determined as described previously (1). All NEIL1 concentrations reported are active, rather than total protein concentrations.

Substrate DNA Preparation. Oligonucleotides containing 8-oxoguanine (OG) were synthesized at the Core Facility at the University of Utah Medical School while those containing Tg were purchased from Midland Reagents. The Gh- and Sp-containing oligonucleotides were prepared by oxidation of OG oligonucleotide (6 nmoles) using 100 μM Na_2IrCl_6 in 100 mM NaCl containing deionized water at room temperature or 60 °C, respectively. The resulting Gh, and two diastereomers of Sp-containing oligonucleotide were separated and isolated by HPLC using a Dionex DNAPac PA-100 as previously described (1). The two diastereomers of Sp are designated Sp1 and Sp2 based on their elution order under these conditions. The appropriate lesion-containing strand was end-labeled with ^{32}P-ATP (PerkinElmer) using T4 polynucleotide kinase (Invitrogen) following the manufacturer's protocol and annealed with the appropriate complementary strands to form the duplex, bulge and bubble substrates (2).

Glycosylase Assays. The glycosylase activity of NEIL1 was evaluated with single-turnover experiments and the relevant rate constants (k_g) determined as reported previously (1, 2). Briefly, 20 nM of substrate DNA was incubated at 37 °C with 200 nM of active enzyme in assay buffer (20 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.1 mg/mL BSA and 150 mM NaCl) and aliquots were removed from the reaction mixture at various time points and quenched with NaOH. For substrates where the reaction was too fast to measure manually (rate constants > 4 min^{-1}), a rapid quench flow instrument (RQF-3) from Kintek was used. Products were resolved via denaturing PAGE. Data analysis was performed using ImageQuant software for quantification of storage phosphor autoradiograms and Grafit 5.0 software for appropriate fitting of the reaction profiles to determine the relevant rate constants.

ADAR Overexpression and Purification. Plasmid pScE[hA1a*-His_6] was used for overexpression of human ADAR1a p110 in *Saccharomyces cereviseae* (3). Plasmid hADAR2a-LV$(H)_6$ was used for overexpression of human ADAR2a in *Saccharomyces cereviseae* (4, 5). ADAR1 and ADAR2 were purified from yeast lysates as previously described (3, 4).

Preparation of RNA Substrate for In Vitro ADAR Assays. DNA was isolated from human glioblastoma U87 (HTB-14) cells (ATCC Manassas, VA) using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The sequence surrounding the NEIL1 recoding site, including 100 nucleotides 5′ and 100 nucleotides 3′ (chromosome 15, 73433039-73433239, hg18), was amplified using the primers: NGF 5′-GCGCGCAA GCTTGTGTAGCTGAGGTCTGGGCC-3′ and NGR 5′-GCG CGCGGATCCATGCCGGTCCTGCAGG-3′. The PCR was carried out with the following program: 98 °C for 30 s; 30 cycles of 98 °C for 10 s, 70 °C for 30 s and 72 °C for 30 s; 72 °C for 7 min; and 4 °C hold. The PCR product was digested with BamHI-HF and HindIII (New England Biolabs, Ipswich, MA, USA) and ligated into a pGE plasmid (Biomatik, Wilmington, DE, USA) digested with BamHI-HF and HindIII. The sequence was confirmed using a T7 promoter primer. The resulting plasmid was linearized with BamHI (New England Biolabs, Ipswich, MA, USA). T7 RNA polymerase was prepared by overexpression in *E. coli.*. In vitro transcription was carried out by incubating the linearized plasmid with T7 RNA polymerase with transcription buffer (80 mM Hepes, 25 mM $MgCl_2$, 2 mM spermidine, 30 mM DTT, pH 7.5), NTPs (8 mM), and ribonuclease inhibitor (Promega, Madison, WI, USA) (1 U/μL) for 1.5 h at 42 °C. This procedure gave an RNA product corresponding to the DNA sequence: 5′-CGAATTGGGTACGAATTCCCCGTACAAGC-TT-(chromosome 15, 73433039-73433239)-G-3′. The product was purified by urea-polyacrylamide gel electrophoresis (PAGE) (5%). After electrophoresis, the RNA bands were visualized by UV shadowing (254 nm light, F254 TLC plate as a backing), and extracted from the gel via the crush and soak method at 4 °C overnight in to 0.5 M NH_4OAc, 0.1% SDS, and 0.1 mM EDTA. Polyacrylamide particles were removed using a Centrex filter (0.2 μm) followed by a phenol-chloroform extraction and ethanol precipitation. The RNA solution was lyophilized to dryness, resuspended in a solution of 1 X TE buffer and 100 mM NaCl and quantified by absorbance measurements at 260 nm. The solution was diluted in the same buffer to 180 nM. The RNA was refolded by heating to 95 °C for 5 min and then allowed to slowly cool to room temperature over 4 h.

Preparation of Mutant RNAs for In Vitro ADAR Assays. Mutant 1 was prepared from the plasmid containing the wild-type sequence using the primers N2DF 5′-TCTCTGCCTGTTCCTCTCGG CCACAGGGGGCAAAGG-3′ and N2DR 5′-CCTTTGCCCCC TGTGGCCGAGAGGAACAGGCAGAGA-3′. Mutant 2 was prepared using the primers N2RF 5′-CCTCTCGGCCACAGGG CTGAAAGGCTACGGGTCAG-3′ and N2RR 5′-CTGACCCG-TAGCCTTTCAGCCCTGTGGCCGAGAGG-3′. Mutant 3 was prepared using the primers NCGF 5′-CTCTGCCTGTTCCT GTGTCCCACAGGGGG-3′ and NCGR 5′-CCCCCTGTGGG ACACAGGAACAGGCAGAG-3′. To make all these mutants, the following PCR program was used: 95 °C for 30 s; 16 cycles of 95 °C for 30 s, 55 °C for 1 min, 68 °C for 2 min; and 68 °C for 7 min; 4 °C hold. The parent plasmid was digested with DpnI (New England Biolabs, Ipswich, MA, USA) and the PCR products were transformed into Z-competent cells (from Zymo Research, Orange, CA, USA, prepared according to the manufacturer's instructions). Plasmids were sequenced for each mutant to confirm the sequence. RNAs were prepared from the mutant plasmids by in vitro transcription as described above for the wild-type sequence.

In Vitro Deamination Assay. Editing of the NEIL1 substrate RNA was evaluated by the following reaction. For the comparison of ADAR1 and ADAR2 editing, ADAR1 or ADAR2 (1 μM final protein concentration) was mixed with 10 nM RNA in assay buffer containing 15 mM Tris-HCl, pH 7.4, 3% glycerol, 0.5 mM DTT, 60 mM KCl, 3 mM $MgCl_2$, 1.5 mM EDTA, 0.003% Nonidet P-40, 160 units/mL RNasin (Promega, Madison, WI, USA), and

1.0 ug/mL yeast $tRNA^{Phe}$. For the comparison of different NEIL1 RNA mutants, the same procedure was used but the final concentration of ADAR1 was 650 nM. Before adding the enzyme, the other reaction components were incubated at 30 °C for 5 min. The reaction was carried out at 30 °C for 3 h. The RNA was purified by phenol-chloroform extraction and ethanol precipitation. RT-PCR was carried out using primers SQF 5′-TGGG TACGAATTCCCCGTACAAGCTT-3′ and NGR (above) and the Access RT-PCR System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The PCR was carried out using the following program: 45 °C for 45 min; 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 60 °C for 1 min and 68 °C for 2 min; 68 °C for 7 min; and 4 °C hold. The extent of editing was determined by DNA sequencing using primer SQF (above). Editing was quantified using 4Peaks (Mekentosj BV, Aalsmeer, The Netherlands) and ImageJ software (NIH, Bethesda, MD) (6).

Cell Culture and mRNA Isolation. Human glioblastoma U87 cells were grown and maintained in DMEM with 10% FBS (Invitrogen, Carlsbad, CA) containing 5.6 mM D-glucose and 4 mM L-glutamine. Cells were lysed according to MicroPoly(A)Purist Kit (Ambion, Austin, TX) protocol and cell lysate was aliquoted for mRNA isolation. Messenger RNA was isolated from cell lysates following manufacturer protocol. Human interferon-α (2A) (IFN-α 2A) was obtained from PBL Biomedical Laboratories (Piscataway, NJ). IFN-α (10^5 U/mL) stocks were made in PBS containing 1% FBS. Cells were grown to ∼70% confluence (10^7 cells), washed, and treated with media containing 10^3 U/mL IFN-α. Cells were cultured an additional 24 h in media containing IFN-α before cell lysis.

Analysis of NEIL1 mRNA Editing in U87 Cells. RNA editing analysis was carried out using RT and nested PCR. Oligonucleotides for RT and PCR were as follows: N1-TCOF 5′-TCCAGACCT GCTGGAGCTAT-3′, N1-TCOR 5′-TGGCCTTGGATTTCTTT TTG-3′, N1-TCIF 5′-CCCAAGGAAGTGGTCCAGTTGG-3′, and N1-TCIR 5′-CTGGAACCAGATGGTACGGCC-3′. Reverse transcription of isolated mRNA was carried out using N1-TCOR primer and AMV reverse transcriptase (Promega, Madison, WI). The RT reaction was performed using 500 nM reverse primer, 500 μM deoxynucleotide triphosphate mix, 20 U RNasin (Promega, Madison, WI), and 5 U AMV RT in AMV RT buffer to 30 μL total reaction volume. The reaction was incubated at 42 °C for 90 min, heat denatured at 95 °C for 2 min, and used as template for nested PCR. All PCRs were carried out using Phusion Hot Start DNA Polymerase (New England Biolabs, Ipswich, MA). PCRs were performed using 600 nM of each primer with 300 nM deoxynucleotide triphosphate mix, and 1 U Phusion polymerase in high-fidelity (HF) buffer to 50 μL final volume. The first PCR utilized outside primers N1-TCOF, N1-TCOR and 1 μL RT reaction with thermocycler conditions of 98 °C for 30 s hot start, followed by 40 cycles of 98 °C for 10 s, 67 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. A volume of 1 μL from the first PCR reaction was carried forward into a nested PCR using inside primers N1-TCIF and N1-TCIR. The PCR conditions were 98 °C for 30 s hot start, followed by 30 cycles of 98 °C for 10 s, 71 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min final extension. PCR products were run on a 1.3% agarose gel. Bands corresponding to 150 bp were cut out, extracted with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and submitted for DNA sequencing (Davis Sequencing, Davis, CA) using reverse primer N1-TCIR. Sequences were analyzed using 4Peaks for A to G changes. Sequencing traces shown are representative of results from two independent experiments.

1. Krishnamurthy N, Zhao X, Burrows CJ, David SS (2008) Superior removal of hydantoin lesions relative to other oxidized bases by human DNA glycosylase hNEIL1. *Biochemistry* 47:7137–7146.
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4. Macbeth MR, Lingam AT, Bass BL (2004) Evidence for auto-inhibition by the N terminus of hADAR2 and activation by dsRNA binding. *RNA* 10:1563–1571.
5. Haudenschild BL, et al. (2004) A transition state analogue for an RNA-editing reaction. *J Am Chem Soc* 126:11213–11219.
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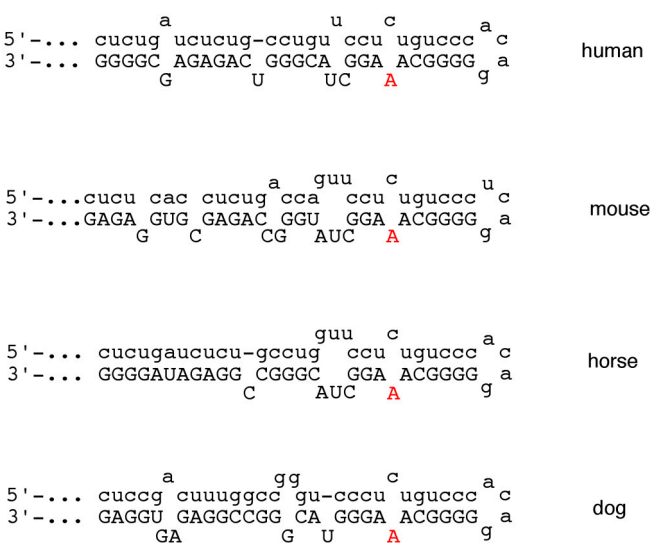

Fig. S1. RNA secondary structure surrounding the NEIL1 recoding site conserved in other vertebrates. NEIL1 gene sequences were obtained from the UCSC Bioinformatics Site (http://genome.ucsc.edu) and folded using *mFold* (1). Human (homo sapiens, hg18), mouse (mus musculus, mm9), dog (canis lupus familiaris, canFam2), horse (equus caballus, equCab2).

1 Zuker M (2003) M-fold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31:3406–3415.

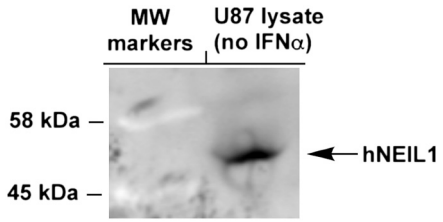

Fig. S2. NEIL1 protein present in lysates of human glioblastoma (U87) cells grown in the absence of IFNα. Western analysis performed with NEIL1 antibody (Calbiochem). Cells were grown as described in the *Cell Culture and mRNA Isolation* section of the *SI Text*, and lysed with lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, protease inhibitors).