SUPPLEMENTARY DATA

NUDT16 is a (deoxy)inosine diphosphatase, and its deficiency induces accumulation of single strand breaks in nuclear DNA and growth arrest

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Supplemental Experimental Procedures

Free Nucleotides

The nucleotides used as substrates for NUDT16 were purchased from Sigma-Aldrich (St Louis, MO, USA), TriLink Biotechnologies (San Diego, CA, USA) or Jena Bioscience (Jena, Germany).

Because the purchased ITP, GTP and dGTP nucleotides contained small amounts of their respective diphosphate forms, we purified them using the following procedure. The nucleotides were separated on a Wakopak Handy ODS column (4.6 mm I.D. x 250 mm L, Wako, Osaka, Japan) using an Alliance photodiode array HPLC system (Waters, Milford, MA, USA) at a flow rate of 0.6 mL/min with HPLC buffer [75 mM triethylammonium hydrogen carbonate (TEAB) pH 7.0]. After TEAB was removed from the collected fractions by five rounds of co-lyophilization with distilled water, the purified nucleotides were dissolved with distilled water and were used for enzymatic reactions.

dIDP was prepared by incubation of the reaction mixture [5 mM dADP, 100 mM sodium nitrite ($NaNO_2$), 500 mM sodium thiocyanate (NaSCN), 3 M sodium acetate ($NaCH_3COO$) pH 3.2] at 37°C for 1 h. The reacted sample was separated on a Wakopak Handy ODS column using an HPLC system, at a flow rate of 0.8 mL/min with HPLC buffer (80 mM formic ammonium, pH 6.4) and then the dIDP fraction was collected. The fraction was lyophilized, dissolved in distilled water, and then used for enzymatic reactions.

Synthetic Oligonucleotides

The synthetic oligonucleotides listed below were purchased from Sigma-Aldrich, Japan and used as PCR primers: 5'NdeI-hNUDT16, 5'-TCCGCATATGGCCGGAGCCCGCAGGCTGG-3'; 3'BamHI-hNUDT16, 5'-CGGATCCTAGTGATGAGCTGGAATCTTAAGGC-3'; 5'NdeI-mNUDT16, 5'-CTCCATATGGAGGGGCATCGGAAAGTGGAG-3'; 3'Hind-mNUDT16, 5'-GTAAGCTTCTACTTAGAATCTGGGATCTTTAG-3'; NUDT16 forward, 5'-CCGCTGCGCTACGCCATACTGAT-3'; NUDT16 reverse, 5'-GCGTGTCCACGAATCCGCCG-3'; 18S rRNA forward, 5'-CTTAGAGGGACAAGTGGCG-3'; 18S rRNA reverse, 5'-GGACATCTAAGGGCATCACA-3'.

Isolation of Human NUDT16 and Mouse Nudt16 cDNAs

Total cellular RNAs were prepared from HeLa MR cells and from C57BL/6J mouse cerebellum using ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). First-strand cDNAs were prepared using PrimeScript reverse transcriptase (Takara, Kyoto, Japan) and oligo dT primer. Human *NUDT16* and mouse *Nudt16* cDNA fragments were amplified by PCR from first strand cDNAs using AmpliTaq Gold DNA polymerase (Takara) and primer sets; 5'NdeI-hNUDT16/3'BamHI-hNUDT16 and 5'NdeI-mNUDT16/3'Hind-mNUDT16 respectively. Amplified fragments were subcloned into pT7Blue-2 T-vector (Merck) to generate the plasmids pT7Blue2T:*NUDT16*(NdeI/BamHI) and pT7Blue2T:*Nudt16*(NdeI/HindIII), respectively. The nucleotide sequences of the inserted cDNA fragments were confirmed by sequencing, using a BigDye terminator v3.1 cycle sequencing kit and a model 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Handling and killing of all animals used in this study were in accordance with the national prescribed guidelines, and ethical approval for the studies was granted by the Animal Experiment Committee of Kyushu University.

Construction of Expression Plasmids

Expression plasmids pET28a(+):*NUDT16* and pET28a(+):*Nudt16* were constructed by transferring *NUDT16* and *Nudt16* cDNA fragments from pT7Blue2T:*NUDT16*(NdeI/BamHI) and pT7Blue2T:*Nudt16*(NdeI/HindIII) into the NdeI/BamHI sites and NdeI/HindIII sites of pET28a(+) (Merck, Darmstadt, Germany), respectively.

Expression and Purification of Recombinant His-NUDT16 Protein

Recombinant NUDT16 with an N-terminal His-tag (His-NUDT16) was expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA, USA) transformed with pET28a(+):*NUDT16*. The cells were suspended in buffer A [50 mM Tris-HCl pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.05% Nonidet P-40 (NP-40), 1 mM 2-mercaptoethanol (2-ME), Complete Protease Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany), 5% glycerol], disrupted by sonication, and clarified by centrifugation at 20,000 x *g* for 30 min at 4°C. His-NUDT16 in the supernatant was purified with TALON beads (Clontech, Palo Alto, CA, USA) and then precipitated using ammonium sulfate, and re-dissolved in buffer B (50 mM Tris-HCl pH 8.0, 5 mM KCl, 5 mM $MgCl_2$, 0.05% NP-40, 1 mM 2-ME, 5% glycerol). The dissolved sample was dialyzed three times against 1 L of buffer B and loaded onto a HiTrap-SP anion exchange column (GE Healthcare, Chalfont St Giles, UK) equilibrated with buffer B. Bound proteins were eluted using a linear gradient of KCl (5–500 mM). Fractions containing His-NUDT16 protein were then applied onto a Superdex 75 HR10/30 size exclusion column (Sigma-Aldrich) equilibrated with buffer C (50 mM Tris-HCl pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 0.05% NP-40, 50% glycerol, 1 mM DTT). The purified His-NUDT16

protein was stored at -80°C.

Anti-NUDT16

As an antigen, His-NUDT16 was expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells transformed with pET28a(+):NUDT16, and purified by metal affinity chromatography with TALON beads. Preparation of anti-NUDT16 rabbit antiserum and affinity purification of anti-NUDT16 was performed as described previously (1,2).

Western Blot Analysis

Protein samples were separated by SDS–PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA) for western blot analysis. The membranes were blocked for 1 h at room temperature in TBST (10 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.1% Tween-20) containing 5% non-fat dried milk. The membranes were incubated in TBST containing 2.0 μg/mL of anti-NUDT16 overnight at 4°C, with gentle shaking. The membranes were washed with TBST and then incubated in TBST containing 0.01% horseradish peroxidase-labeled protein A (GE healthcare) for 1 h at room temperature. After washing with TBST, the antibodies bound to each blot were detected by the chemiluminescence method with an Immobilon Western Chemiluminescent HRP Substrate (Millipore). Digitized images were obtained with a LAS1000 Plus (Fuji Film, Tokyo, Japan) and processed for publication using the Adobe Photoshop 5.0 software package (Adobe Systems, San Jose, CA, USA) (2).

Cell Culture

SH-SY5Y cells were purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). HeLa MR cells were routinely grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen, Carlsbad, CA, USA). Both cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

Cell Proliferation Assay

Cell proliferation was determined with a Cell Counting Kit-8 using WST-8 reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. This assay is similar to the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were seeded at a density of 500 cells per well in 96-well plates. After cultivation, WST-8 reagent was added to each well and cells were incubated for an additional 4 h. Absorbance at 450 nm was determined using a Spectra Thermo microplate reader (Tecan, Salzburg, Austria).

Hoechst 33342/PI Assay

Cell death was assessed with Hoechst 33342/PI double staining (3,4). Transfected HeLa MR cells were incubated with 5 μ M Hoechst 33342 (Invitrogen) and 2 μg/mL PI for 20 min and examined under an Axiovert 25CFL fluorescence microscope (Carl Zeiss), equipped with a DP11 digital camera (Olympus, Tokyo, Japan).

Real-Time Quantitative RT-PCR

All total RNAs from human tissues were purchased from Clontech. Total RNAs from HeLa MR cells were isolated with ISOGEN RNA extraction reagent. The relative level of *NUDT16* mRNA was determined by real-time quantitative RT-PCR, according to a previously described method (5). Briefly, 20 μg of each total RNA was treated with 10 U RNase-free DNase I (Roche Applied Science) at 37°C for 1 h, and then purified by phenol/chloroform treatment and ethanol precipitation. cDNA was synthesized from 2 μ g DNA-free total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) using random primers in a total volume of 20 μL. Real-time quantitative PCR was performed to measure the levels of *NUDT16* mRNA and 18S rRNA using an ABI Prism 7000 sequence detection system (Applied Biosystems) with 10 ng cDNA, 200 nM primers and Power SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 μL. The PCR reaction was performed as follows: a single cycle of 50°C for 2 min, a single cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Two primer sets, NUDT16 forward/NUDT16 reverse and 18S rRNA forward/18S rRNA reverse, were designed using primer express software (Applied Biosystems) and their sequences are described above. The specificity of the PCR products was established by dissociating curve analysis, and by running the products on a 2% agarose gel to verify their size. Serial dilutions of a cDNA sample were used to obtain a standard curve for each transcript. The *NUDT16* mRNA levels were normalized to the 18S rRNA levels, and expressed relative to the control sample.

Comet assay

Nuclear DNA fragmentation was monitored using a Comet assay™ kit (Trevigen, Gaithersburg, MD, USA) under alkaline and neutral conditions, according to the manufacturer's instructions. HeLa MR cells were transfected with *NUDT16* or negative control siRNAs. After incubation for 3 days, the cells transfected with *NUDT16* siRNA or negative control siRNA were separately embedded in soft agarose on glass slides and then subjected to the assay. Comet images from 15 – 30 cells for each siRNA were captured using an Axioskop 2 plus equipped with an AxioCam, and were analyzed using the CASP (Comet Assay Software Project) program (6) to quantify tail moment. The assays were independently performed three times independently.

SUPPLEMENTAY REFERENCES

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Optimization of reaction conditions for IDP hydrolysis by His-NUDT16. The dependencies of His-NUDT16 activity on temperature (*A*), buffer pH (*B*), divalent metal cations (*C*) and salts (*D*) were analyzed. The amounts of IMP product were measured and relative ratios are shown. The highest values were detected at (*A*) 60°C, (*B*) 25 mM TAPS buffer pH 9.0, (*C*) 5 mM Mg^{2+} and (*D*) 500 mM KCl.

Supplementary Figure S2. NUDT16 produces free phosphates during hydrolysis of IDP to IMP, but not during hydrolysis of ITP to IMP. IDP or ITP (200 μM) were incubated in reaction mixture with 200 nM His-NUDT16 at 37°C for 4 h (IDP) or 2 h (ITP). IMP and free phosphates were determined with HPLC and with Biomol Green reagent, respectively. Data are mean ± SD from three independent experiments. ND, not detected.

Supplementary Figure S3. Recombinant mouse NUDT16 hydrolyzes dIDP. Recombinant mouse NUDT16 protein with an N-terminal His-tag was expressed in Rosetta-gami B(DE3) *E. coli* (Merck) transformed with pET28a(+):mNudt16 (see Supplemental Experimental Procedures section). *E. coli* with pET28a(+) was used as a vector control. Cell extract was prepared from each bacterial culture, and one μg protein from each cell extract was incubated with 300 μM of dITP substrate in reaction buffer (25 mM Tris-HCl pH 8.5, 100 mM KCl, 5 mM $MgCl_2$, 0.05% NP-40 and 1 mM DTT) for 10 min at 30°C. The products were analyzed by HPLC equipped with a C18 SunFire column (Waters). HPLC charts of absorbance at 249 nm for the products of reaction buffer (upper panel), control extract (middle panel), or extract containing mouse NUDT16 protein (lower panel), are shown.

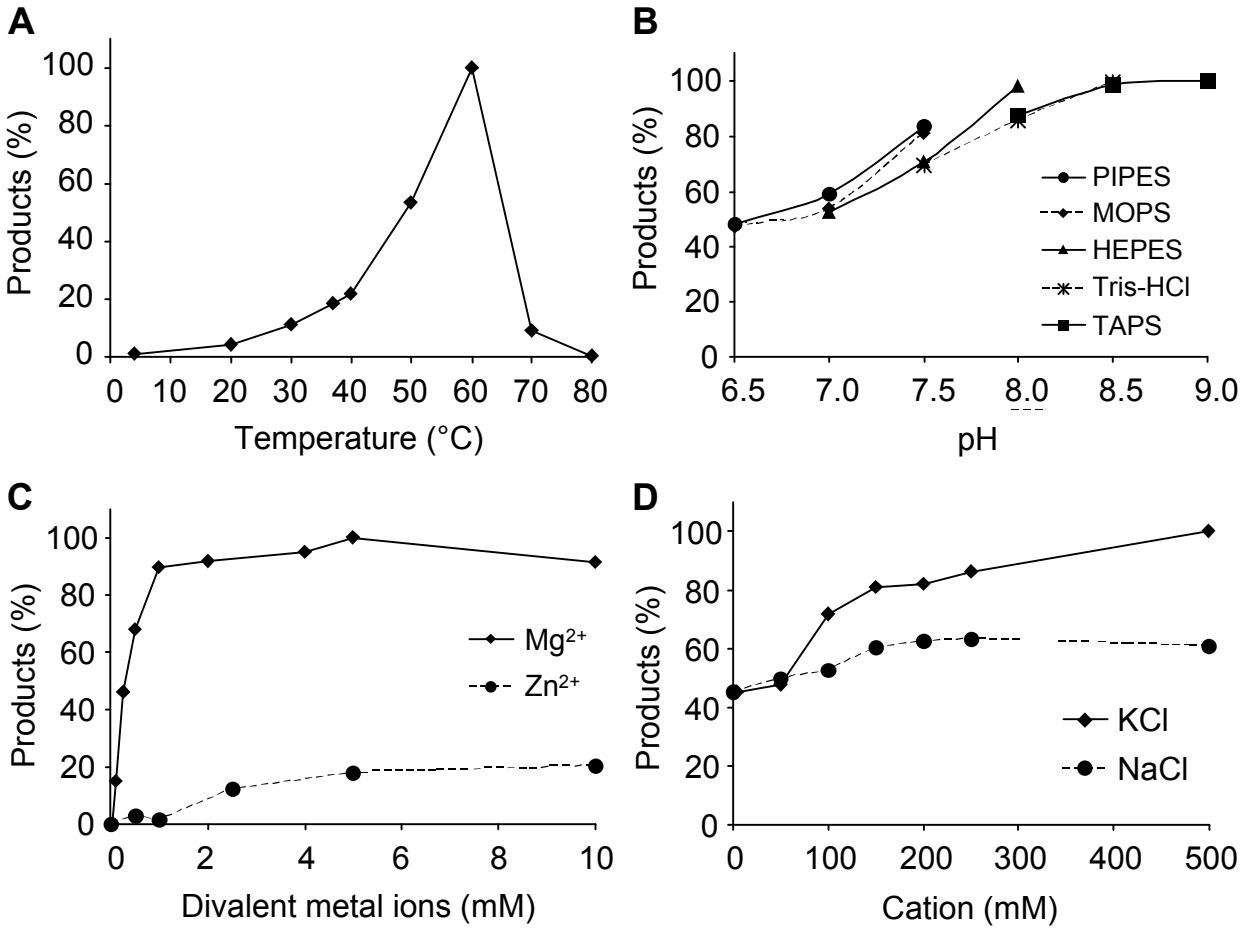
Supplementary Figure S4. Ubiquitous expression of *NUDT16* mRNA in various human tissues. *NUDT16* mRNA levels were determined by real time quantitative RT-PCR. Serially diluted cDNA was used to obtain a standard curve for each transcript. The levels of *NUDT16* mRNA were normalized to that of 18S rRNA in each tissue and its levels relative to that in lung are shown. The levels of relative expression represent the mean ± SD of three measurements.

Supplementary Figure S5. Knockdown of *NUDT16* with a second *NUDT16* siRNA also suppresses proliferation of HeLa MR cells. (*A*) *NUDT16* mRNA levels in HeLa MR cells transfected with a second *NUDT16* siRNA were remarkably suppressed. Three days after transfection with *NUDT16* siRNA#2 or with control siRNA#2, the cells were harvested and total RNAs purified. The RNA samples were analyzed by real-time quantitative RT-PCR for *NUDT16* mRNA and its level relative to that of the control siRNA#2 transfection is shown. (*B*) The cells transfected with *NUDT16* siRNA#2 or with control siRNA#2 were subjected to the cell proliferation assay (see Supplemental Experimental Procedures section). Data are the mean ± SD of three independent siRNA transfections. $*P < 0.0001$ vs. the cells transfected with control siRNA#2, as

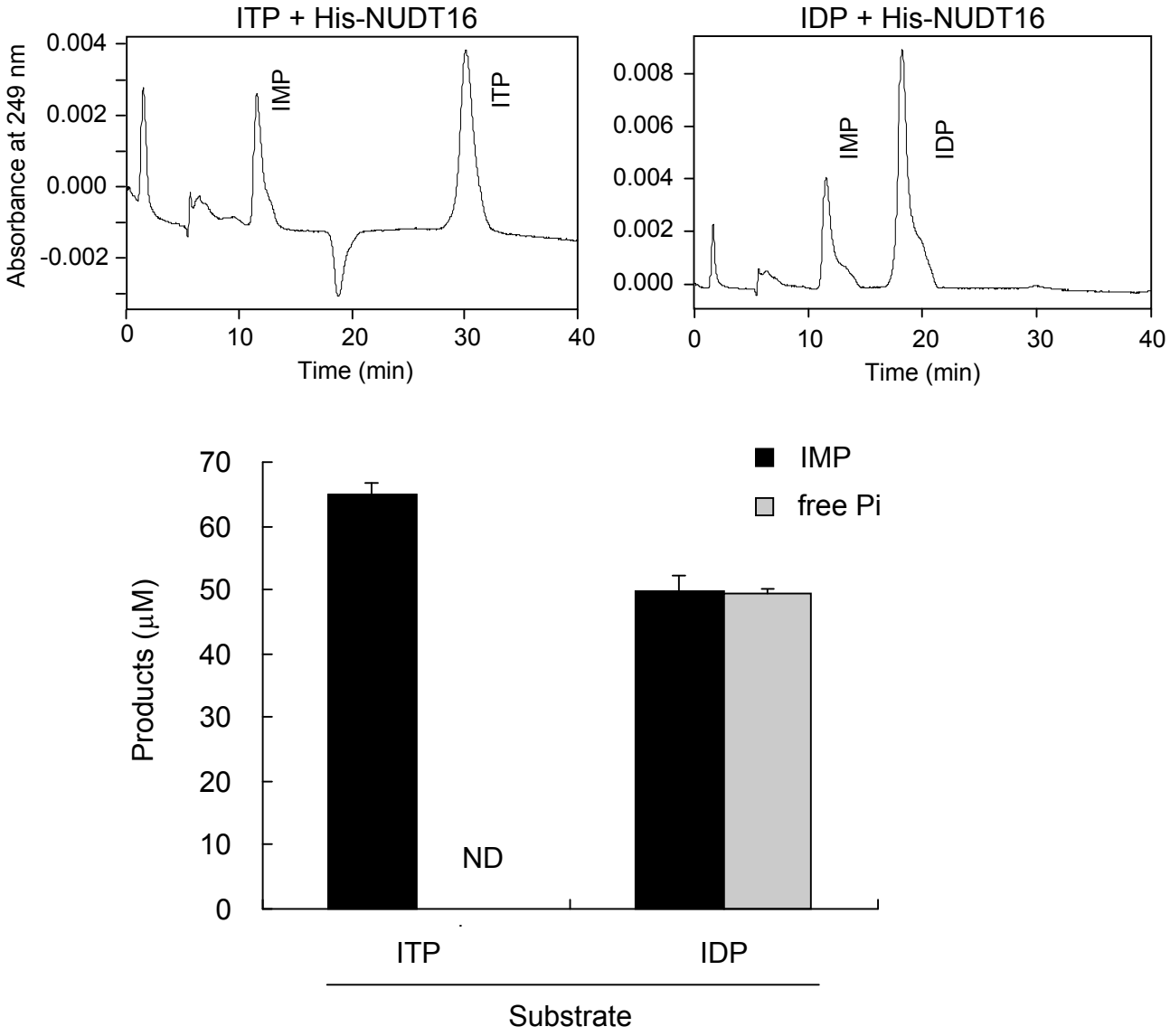
determined by two-way repeated measures ANOVA.

Supplementary Figure S6. Hoechst/PI staining of cells transfected with *NUDT16* siRNA#1 (a,b) or with control siRNA#1 (c,d) were performed to evaluate cell death. As a positive control, HeLa MR cells treated for 24 h with 1 µM staurosporine (e,f) or with 500 µM H_2O_2 (g,h) were used. Fluorescent images of the same microscopic field were obtained using a fluorescence microscope equipped with Zeiss Filterset 15 for Hoechst 33342 and PI signals (left panels; a,c,e,g) or with Zeiss Filterset 01 for PI signal (right panels; b,d,f,h).

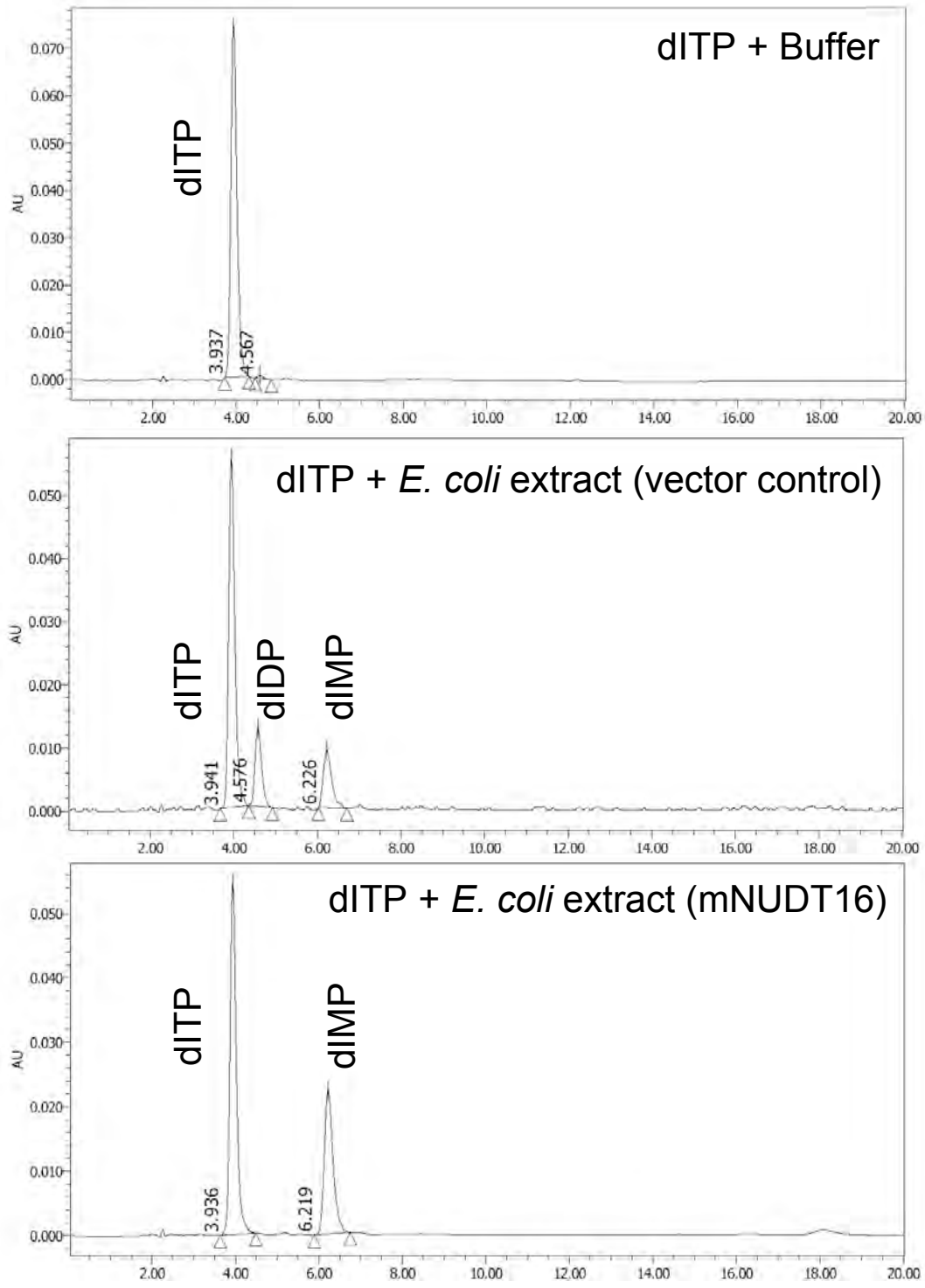
Supplementary Figure S7. Knockdown of *NUDT16* in HeLa MR cells induced single strand breaks, but not double strand breaks in nuclear DNA. Representative comet assay images under alkaline (**A**) or neutral conditions (**B**) are shown for each siRNA in three (1, 2 and 3) independently repeated experiments. Cells treated with H_2O_2 were analyzed as a positive control. **(C)** Box-and-whisker plots of comet assay tail moments under neutral conditions are shown. Tail moments of at least 15 cells were calculated for each group. Mann-Whitney U-test, $P < 0.05$.



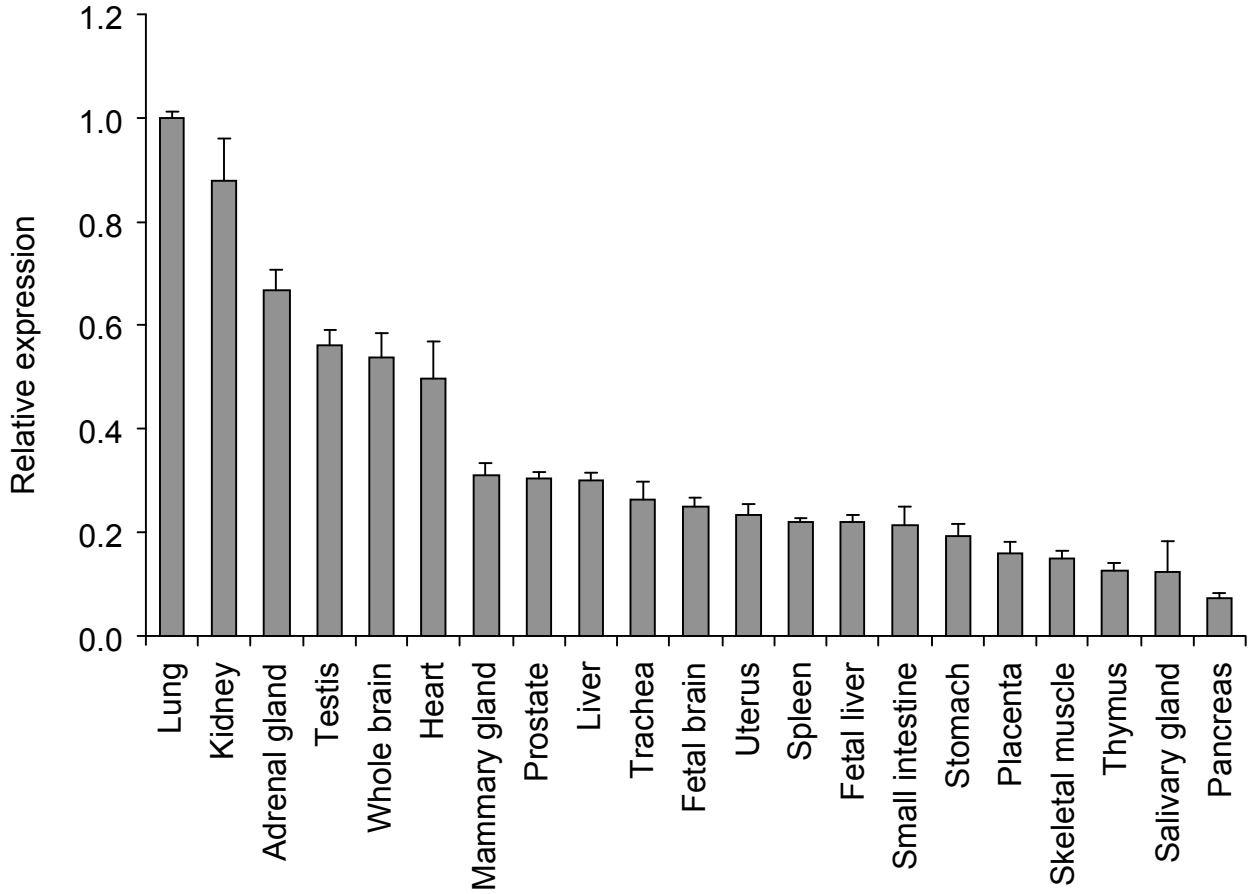
Iyama *et al*., Supplementary Figure 1.



Iyama *et al*., Supplementary Figure 2.



Iyama *et al*., Supplementary Figure 3.



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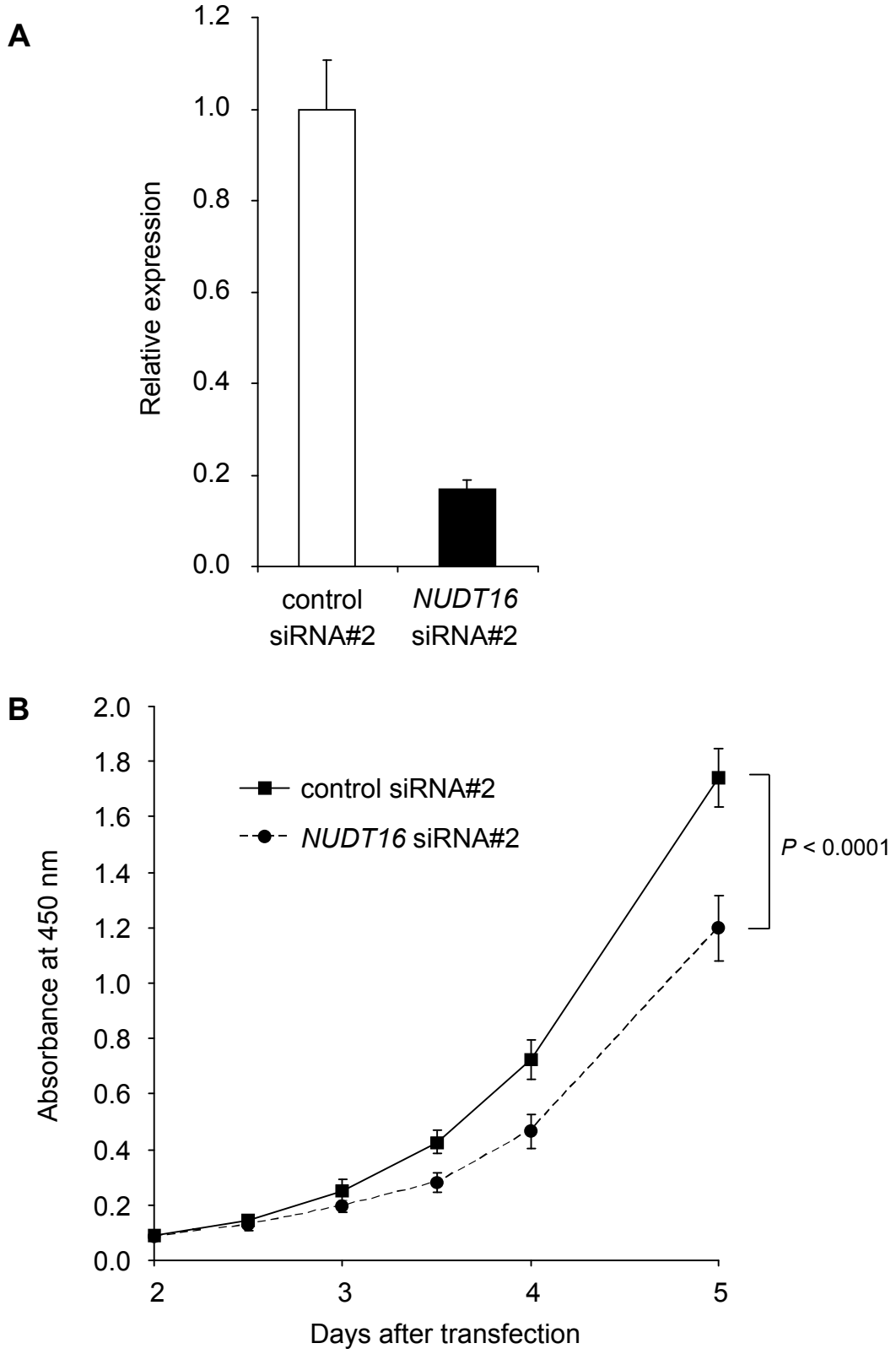

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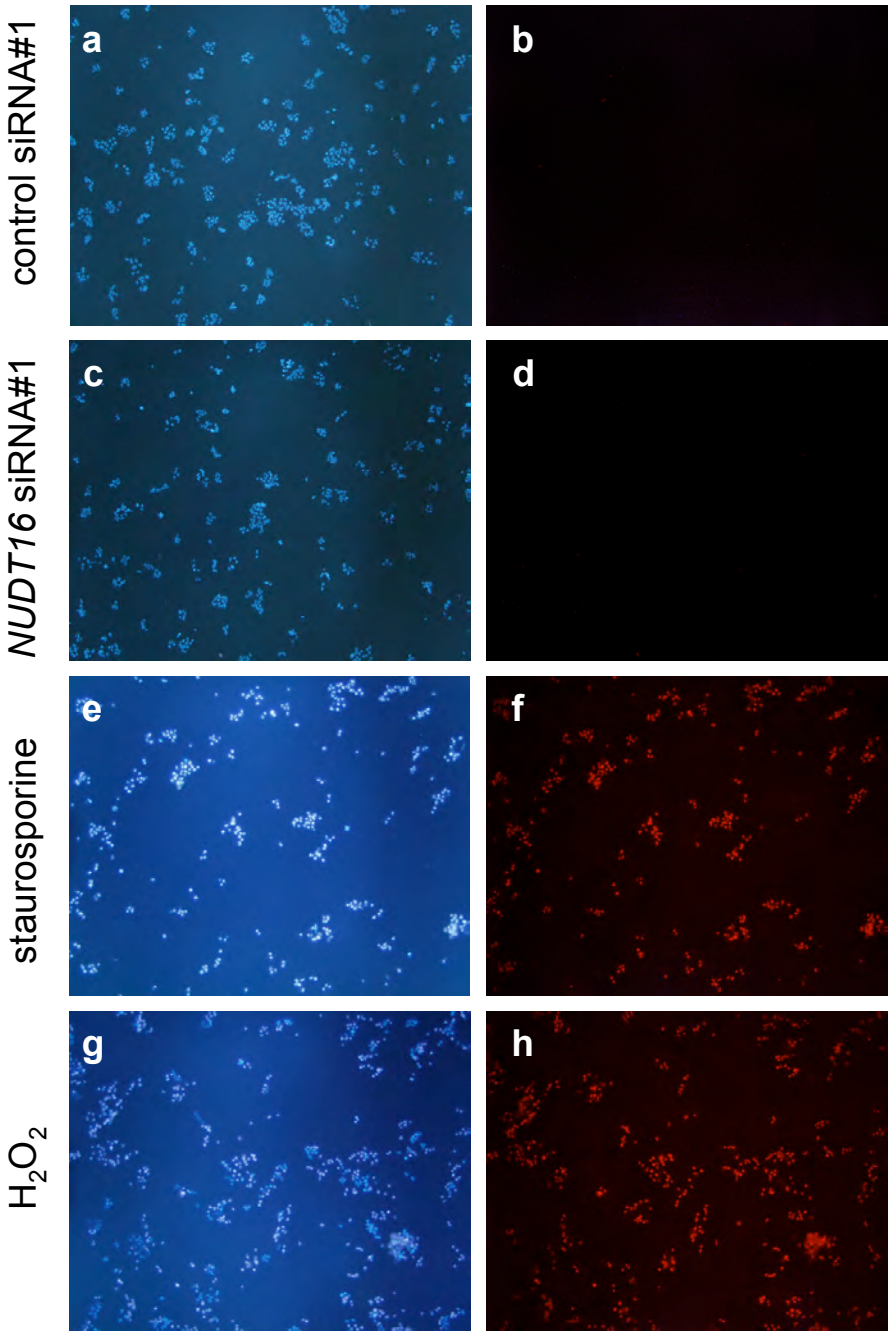

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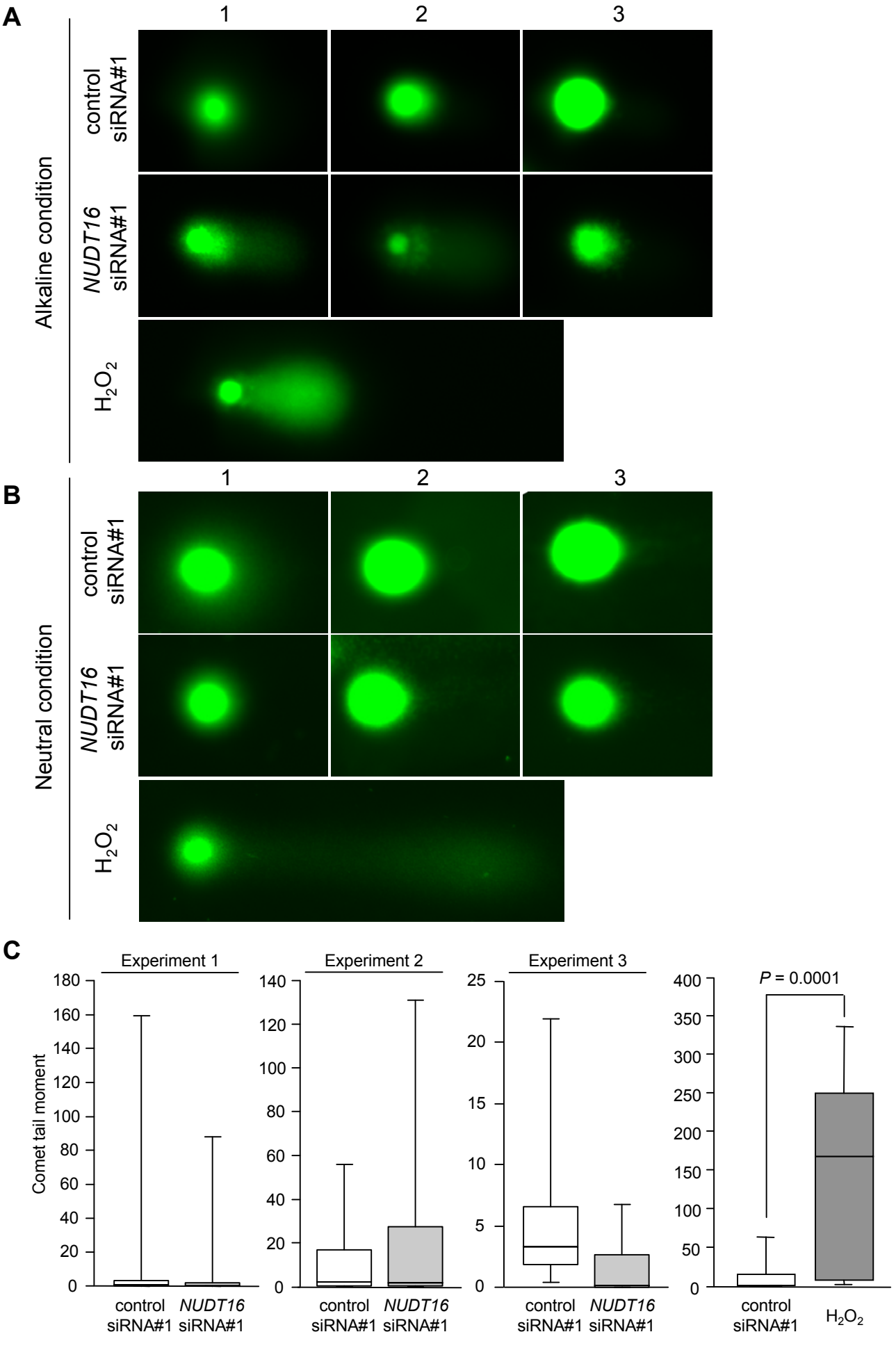

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Supplementary Table S1 Number of peptides matched with NUDT16

Immobilized NTP	Phosphate-Sepharose	Ribose-Sepharose
GTP	0	6
ATP	0	0
XTP	17	20
ITP	14	3
8-oxo-GTP	0	0
2-OH-ATP	0	0
none*	0	

*Sepharose carrier matrix