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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ABSTRACT

Nucleotides function in a variety of biological reactions; however, they can undergo various chemical modifications. Such modified nucleotides may be toxic to cells if not eliminated from the nucleotide pools. We performed a screen for modified-nucleotide binding proteins and identified human nucleoside diphosphate linked moiety X-type motif 16 (NUDT16) protein as an inosine triphosphate (ITP)/xanthosine triphosphate (XTP)/GTP-binding protein. Recombinant NUDT16 hydrolyzes purine nucleoside diphosphates to the corresponding nucleoside monophosphates. Among 29 nucleotides examined, the highest $k_{cat}/K_m$ values were for inosine diphosphate (IDP) and deoxyinosine diphosphate (dIDP). Moreover, NUDT16 moderately hydrolyzes (deoxy)inosine triphosphate ([d]ITP). NUDT16 is mostly localized in the nucleus, and especially in the nucleolus. Knockdown of NUDT16 in HeLa MR cells caused cell cycle arrest in S-phase, reduced cell proliferation, increased accumulation of single-strand breaks in nuclear DNA as well as increased levels of inosine in RNA. We thus concluded that NUDT16 is a (deoxy)inosine diphosphatase that may function mainly in the nucleus to protect cells from deleterious effects of (d)ITP.

INTRODUCTION

Intracellular free nucleotides play essential roles as precursors in the synthesis of DNA and RNA, and as molecules for energy storage, cofactors of metabolic pathways and regulators of signal transduction. Free nucleotides can, however, undergo various chemical modifications by endogenous and exogenous reactive molecules, some of which are inevitably produced in living cells. Chemical modifications may alter the properties of nucleotides, including their interaction with other molecules (1). Some modified deoxynucleotides are known to be incorporated into and to accumulate in newly synthesized DNA during DNA replication. Modified nucleotides, accumulated in either the nucleotide pool or DNA, may inhibit DNA or RNA polymerases during replication or transcription, reduce polymerase fidelity or alter the DNA structure, thus resulting in mutagenesis and carcinogenesis (2,3), cell death and degenerative disorders (4,5) or senescence and aging (6). In addition to DNA metabolism, the other biological functions of canonical nucleotides may also be adversely affected by modified nucleotides. Therefore, because modified nucleotides are constantly generated under physiological conditions, it is crucially important to understand how they are eliminated from cells.

It had been established that cells are equipped with specific enzymes to hydrolyze modified nucleoside triphosphates to the corresponding monophosphates to avoid their deleterious effects (4,7). Deoxyuridine triphosphatase (dUTPase), for example, hydrolyzes dUTP, thus preventing its incorporation into DNA. We have previously demonstrated that MTH1 hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-2'-deoxyguanosine triphosphate (8-oxo-dGTP) or 8-oxoGTP and prevents their incorporation into DNA or RNA (4).

Deamination of purine bases is one of the major chemical modifications that occurs to nucleotides under physiological conditions (8). Deamination of adenine at C6 or guanine at C2 generates hypoxanthine or xanthine, respectively, suggesting that (deoxy)inosine triphosphate ([d]ITP) and (deoxy)xanthosine triphosphate ([d]XTP) can be generated from (d)ATP and (d)GTP, respectively.

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respectively. Moreover, IMP is abundant in cells as a normal intermediate of de novo synthesis of purine nucleotides (9), and most cells can generate IDP or ITP from IMP (10). If IDP is converted to dIDP by ribonucleotide reductase, increased dITP levels will result (11). Incorporation of such deaminated purine nucleotides into DNA or RNA causes genomic mutations or synthesis of abnormal proteins because hypoxanthine and xanthine can mis-pair with cytosine or thymine (12,13).

In human and rodents, ITPA, an inosine triphosphatase (ITPase), has been reported to hydrolyze (d)ITP and XTP to the corresponding nucleoside monophosphates and pyrophosphates (14,15). We have previously reported that Itpa knock- out (KO) mice die before weaning with features of growth retardation and heart failure. In addition, these mice show accumulation of IMP in cellular RNA in various tissues or accumulation of ITP in the nucleotide pool of erythrocytes (16). The heart failure in Itpa-KO mice suggests that an accumulation of ITP in the nucleotide pool might impair some functions of adenosine triphosphate (ATP), such as ATP-dependent actomyosin contraction (17). In humans, some variants of ITPA are reported to be associated with decreased ITPase activity (18,19). In erythrocytes of ITPA-deficient individuals, it was established that the level of ITP, which is not detected in normal individuals, is increased to a detectable level, as observed in Itpa-KO mice. ITPA deficiency in patients with inflammatory bowel disease is, however, likely to be related to azathioprine intolerance, but does not cause any severe phenotype (19,20). To date, however, likely to be related to azathioprine intolerance, it is not known why ITPA deficiency causes a severe phenotype in mouse but not in humans. We, therefore, hypothesized that human cells are equipped with a compensatory mechanism which can efficiently suppress the ITPA deficiency.

In the present study, to identify novel ITP hydrolyzing enzymes or proteins that target ITP, we performed a comprehensive screen of proteins that specifically bind to ITP or enzymes or proteins that target ITP, we performed a comprehensive screen of proteins that specifically bind to ITP or XTP. For this purpose, we performed a comprehensive screen of proteins that specifically bind to ITP or XTP. For this purpose, we performed a comprehensive screen of proteins that specifically bind to ITP or XTP.

## MATERIALS AND METHODS

### Purification and identification of modified nucleotide-binding proteins

Modified nucleotide-binding proteins were purified and identified with pull-down assays as follows. \( \gamma \)-amino-octyl-nucleoside 5'-triphosphate-Sepharose (phosphate-NTP Sepharose) and/or 2'/3'-O-(2-aminoethyl-carbamoyl)-nucleoside 5'-triphosphate-Sepharose (ribose-NTP Sepharose) for GTP, ATP, XTP, ITP, 8-oxo-GTP and 2-OH-ATP were purchased from Jena Bioscience (Jena, Germany) and used for pull-down assays. The cell extract of SH-SY5Y cells was prepared by sonication of cells in lysis buffer [1 ml for 5 × 10⁷ cells, 25 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.05% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), 1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)] and clarified by centrifugation, as described previously (21).

Protein concentration in the supernatant was measured with a DC-protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. Twenty microliters of each phosphosphate-NTP Sepharose, ribose-NTP Sepharose, and Sepharose carrier matrix were individually suspended in 1 ml of the supernatant, incubated for 15 min at 4°C, and washed three times with the lysis buffer without protease inhibitor cocktail. Bound proteins in each pulled-down sample were eluted with 40 µl of 2 × SDS sampling buffer (Sigma-Aldrich, St Louis, MO, USA), separated by SDS-PAGE, stained by silver staining with EzStain Silver kit (ATTO Co., Tokyo, Japan), and analyzed by LC-MS/MS, as described previously (21). Collision-induced dissociation spectra were acquired and compared with those in the International Protein Index (IPI version 3.26; European Bioinformatics Institute, Hinxton, UK) using the MASCOT search engine (Matrix Science, Boston, MA, USA). The high-scoring peptide sequences (MASCOT score >45) assigned by MASCOT were manually confirmed by comparison with the corresponding collision-induced dissociation spectra. Finally, we selected as candidate proteins those proteins for which multiple peptides were identified in this analysis.

### Nucleotide-hydrolyzing assay with His-NUDT16

Each substrate nucleotide was incubated in reaction buffer (25 mM Tris–HCl pH 7.5, 150 mM KCl, 5 mM MgCl₂, 0.01% NP-40, 100 µg/ml BSA, 1 mM DTT) for 10 min at 37°C. Then, an equal volume of reaction buffer containing 100 µM recombinant NUDT16 with a His-tag at the N terminus (His-NUDT16), was mixed with the substrate solution. The mixture was further incubated at 37°C for 0–60 min, and then mixed with ice-cold EDTA to a final concentration of 50 mM to stop the reaction. The reaction products were characterized by centrifugation at 9000g for 5 min at 4°C, and then separated on a Wako Hardy ODS column (Wako, Osaka, Japan) or on a TSK gel DEAE-2SW column (Tosoh, Tokyo, Japan) using an HPLC system, at a flow rate of 0.6 ml/min with HPLC buffer 1 (0.1 M potassium phosphate buffer pH 4.0) or at 0.8 ml/min with HPLC buffer 2 (75 mM sodium phosphate buffer pH 6.4, 5% acetonitrile, 0.4 mM EDTA). Nucleotides were quantified by ultraviolet (UV) absorption. Kinetic parameters, \( k_{cat} \) and \( K_{m} \), were calculated by a fit of the velocity data to the Michaelis–Menten equation using the SigmaPlot analysis software version 11 with Enzyme Kinetics Module 1.3 (Systat Software, San Jose, CA, USA).

Free phosphates were quantified colorimetrically with a modified Malachite Green phosphate detection method using Biomer reagent (Enzo Life Sciences International, Plymouth Meeting, PA, USA) (22,23). One-hundred microliters of the Biomer reagent was added to 50 µl of each reaction mixture, and the mixture was incubated for 30 min at room temperature. The change in absorbance at 620 nm was measured and used to determine free phosphate concentrations by comparison with a standard curve.
siRNA and transfection

All siRNA oligonucleotides used in this study, NUDT16 siRNA#1 (Silencer Select NUDT16 siRNA; #s43642), NUDT16 siRNA#2 (Silencer NUDT16 siRNA; #s38731), control siRNA#1 (Silencer Select Negative Control #1 siRNA, Cat#4390844) and control siRNA#2 (Silencer Negative Control #1 siRNA, Cat#AM4635) were purchased from Applied Biosystems (Foster City, CA, USA). HeLa MR cells were transfected with siRNAs by electroporation using a Microporator-Mini (Digital Bio Technology, Seoul, Korea), according to the manufacturer’s instructions. In brief, 10⁵ cells were suspended in 10µl of R buffer (provided in the MicroPoration kit) and mixed with 1µl of one of the siRNAs (50 µM) before electroporation. The transfected cells were suspended in fresh culture medium. After incubation for 24 h, the cells were resuspended in new culture dishes at a density of 1.515 × 10⁴/cm². After an additional incubation, the cells were subjected to further assays.

Immunofluorescence microscopy for NUDT16 and single-stranded DNA

To perform immunofluorescence microscopy for NUDT16 and single-stranded DNA (ssDNA), HeLa MR cells were seeded onto Lab-Tek two-well chamber slides (Thermo Fisher Scientific, Rockford, IL, USA), 24 h after transfection with control siRNA#1 or NUDT16 siRNA#1. The cells were further cultured for 48 h and fixed with 4% paraformamide in phosphate buffered saline (PBS) containing 0.1% Triton X-100. The fixed cells were treated with anti-NUDT16 or with anti-nucleolin (sc-8031, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in combination with Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11034, Invitrogen, Carlsbad, CA, USA) or Alexa Fluor 594-conjugated goat anti-mouse IgG (A-11035, Invitrogen). Nuclei were counterstained with 4′-diamino-2-phenylindole (DAPI) (50 ng/ml; Vector Scientific). The slide was observed under an Axio Imager A1 plus equipped with AxioCam and AxioVision software (Carl Zeiss). A total of 30 cells in metaphase were examined for each preparation.

Quantification of deoxyinosine or inosine by LC-MS/MS

The DNA deoxyinosine or RNA inosine levels were determined as follows. The preparation and digestion of nuclear DNA samples were performed according to methods described previously (26), except that 10 mM 2, 6, 6-tetramethylpiperidine-N-oxyl (TEMPO, Wako) and 20 µM 2′-deoxycoformycin, an adenosine deaminase inhibitor, kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), were added to all reagents at all stages of manipulation, according to the method described by Taghizadeh et al. (27). RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) in the presence of 20 mM TEMPO and 20 µM 2′-deoxycoformycin. DNA or RNA samples were digested with Nuclease P1 (Yamasu, Chiba, Japan) and alkaline phosphatase (Sigma-Aldrich) in the presence of 20 mM TEMPO and 20 µM 2′-deoxycoformycin, and digested samples were subjected to LC-MS/MS analysis using the Shimadzu VP-10 HPLC system connected to the API3000 MS/MS system (PE-SCIEX), as described previously (26).

Cell cycle analysis

Flow cytometric analysis of the cell cycle was performed as previously described (28,29). Briefly, 1 × 10⁶ cells were suspended in 1 ml PBS containing 0.2% Triton X for the naked nuclei preparation. Then, the cell suspension was passed through a nylon mesh membrane. Five microliters of RNase A (1 mg/ml) and 50 µl of propidium iodide (PI) (1 mg/ml) were then added to the suspension. DNA content and cell numbers were analyzed with an LSR flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with CellQuest and ModFit software (Becton Dickinson).

Karyotype analysis

Fifty-percent confluent cultures of HeLa MR cells were treated with 0.1 µg/ml colcemid (Nacalai Tesque) for 30 min and then harvested. After hypotonic treatment (75 mM KCl), cells were fixed in freshly prepared Carnoy’s fixative (methanol:acetic acid; 3:1), and the cell suspension was dropped onto a glass slide, air-dried and immediately stained with freshly prepared Giemsa staining solution (Merck, 25 × diluted in PBS) for 20 min. After rinsing the slide twice in PBS and twice in distilled water, a cover slide was mounted onto the air-dried slide with Permount mounting medium (Thermo Fisher Scientific). The slide was observed under an Axio Imager.A1 plus equipped with AxioCam and AxioVision software (Carl Zeiss). A total of 30 cells in metaphase were examined for each preparation.

Statistical analysis

All results are expressed as the mean ± SD. Statistical analysis was performed using Stat View 5.0 (SAS Institute, Cary, NC, USA) and each method of statistical analysis is shown in detail in the figure legends. P < 0.05 was considered statistically significant.

Supplementary materials and methods

Descriptions of the following materials and procedures are provided in Supplemental Experimental Procedures: free nucleotides, synthetic oligonucleotides, isolation of human NUDT16 and mouse Nudt16 cDNAs, construction of...
expression plasmids, expression and purification of recombinant His-NUDT16 protein, anti-NUDT16, western blot analysis, cell culture, cell proliferation assay, Hoechst 33342/PI assay, real-time quantitative RT-PCR, and comet assay.

RESULTS

NUDT16 selectively binds to ITP, XTP and GTP

To search for ITP-binding proteins, we purified proteins from whole-cell extracts prepared from SH-SY5Y cells using a pull-down method incorporating various NTP-immobilized Sepharose beads (Figure 1A). The purified proteins were fractionated by SDS-PAGE and visualized by silver staining. The proteins in the gel were digested with trypsin and subjected to LC-MS/MS analysis (see ‘Materials and Methods’ section). By comparing retrieved proteins with multiple peptide sequences among all samples, we identified peptide fragments derived from NUDT16 only in the samples bound to phosphate/ribose-ITP, phosphate/ribose-XTP and ribose-GTP Sepharose beads, but not in samples bound to any other NTP Sepharose beads (Figure 1B and Supplementary Table S1). Western blot analysis of pull-down samples, prepared independently of the above samples using anti-NUDT16, confirmed the LC-MS/MS results by identifying the same nucleotide-binding 20 kDa NUDT16 protein (Figure 1C).

NUDT16 is a (deoxy)inosine diphosphatase

Because NUDT16 is a member of the nudix family of proteins, including the nucleoside triphosphatase MTH1 (NUDT1), we performed biochemical analysis of nucleotide hydrolyzing activity using recombinant NUDT16 protein. His-NUDT16 was expressed in *Escherichia coli* (*E. coli*) and then purified. Samples from each purification step were subjected to SDS-PAGE and a His-NUDT16 polypeptide of ~23 kDa, (its calculated molecular weight is 23.45 kDa) was purified to near homogeneity after size exclusion column chromatography (Figure 2A).

To obtain an overview of substrate specificity for NUDT16, we incubated His-NUDT16 with various nucleotides at 10 or 100 μM (Figure 2C). The products were then analyzed and quantified by HPLC. His-NUDT16 hydrolyzed nucleoside triphosphates/diphosphates (NTPs/NDPs) to the corresponding nucleoside monophosphates (NMPs), and had substrate preferences for purine nucleotides. Especially at substrate concentrations of 10 μM, ITP, dITP, XDP, GDP, dGDP, IDP and dIDP were efficiently hydrolyzed. In contrast, His-NUDT16 did not generate any hydrolyzed product from 7-Me-GDP, ATP, CTP, UTP, 2-OH-ATP, dATP, dCTP, dUTP, TTP nor 2-OH-dATP, even at 100 μM. Because these substrates were not hydrolyzed, they are not represented in Figure 2C.
His-NUDT16 for 1 h at 37°C. Generation of free phosphates (Pi) in these reactions, we analyzed the products using the Malachite Green phosphate detection method to distinguish Pi from pyrophosphates (PPi). Hydrolysis of IDP by His-NUDT16 generated only dIMP from dITP. In contrast, E. coli extract with His-mNUDT16 generated only dIMP from dITP (Supplementary Figure S3). Thus, we concluded that mouse NUDT16 also hydrolyzes dIDP to dIMP.

Expression of NUDT16 in human cell lines and tissues
We determined the levels of NUDT16 mRNA in 21 human tissues by real-time quantitative RT-PCR (Supplementary Figure S4). NUDT16 mRNA was detected in all tissues examined and the highest expression was observed in lung and kidney. Next, we examined levels of NUDT16 mRNA and protein in HeLa MR cells with or without expression of His-mNUDT16. E. coli extract without His-mNUDT16 generated dIDP and dIMP from dITP. In contrast, E. coli extract with His-mNUDT16 generated only dIMP from dITP (Supplementary Figure S3). Thus, we concluded that mouse NUDT16 also hydrolyzes dIDP to dIMP.

Table 1. Kinetic parameters of His-NUDT16

| Substrate | $K_m$ ($\mu$M) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (10$^3$ s$^{-1}$ M$^{-1}$) | Goodness-of-curve fit $R^2$ |
|-----------|---------------|----------------------|--------------------------------------|------------------|
| IDP       | 0.062         | 0.931                | 251                                  | 0.973            |
| dIDP      | 0.088         | 0.966                | 183                                  | 0.990            |
| GDP       | 0.330         | 0.518                | 26.1                                 | 0.987            |
| dGDP      | 0.319         | 0.492                | 25.7                                 | 0.988            |
| XDP       | 15.7          | 2.60                 | 2.76                                 | 0.978            |
| ITP       | 22.1          | 3.06                 | 2.31                                 | 0.980            |
| dITP      | 24.1          | 3.20                 | 2.21                                 | 0.999            |

Because His-NUDT16 efficiently hydrolyzed ITP, dITP, XDP, GDP, dGDP, IDP and dIDP, we performed a detailed analysis of the hydrolysis kinetics for these substrates (Table 1). Fitting Michaelis–Menten type kinetics to the initial rates of reaction revealed a positive correlation for each substrate. Among the substrates, the $k_{cat}/K_m$ values for IDP and dIDP were 251 x 10$^3$ and 183 x 10$^3$ s$^{-1}$ M$^{-1}$, respectively. These were at least seven times higher than the $k_{cat}/K_m$ value for GDP (26.2 x 10$^3$ s$^{-1}$ M$^{-1}$), which was the third highest among the substrates analyzed. IDP and dIDP were, therefore, identified as the best substrates for His-NUDT16.

We also expressed mouse His-tagged NUDT16 protein (His-mNUDT16) in E. coli and analyzed dITP hydrolyzing activity using extracts prepared from E. coli cells with or without expression of His-mNUDT16. E. coli extract without His-mNUDT16 generated dIDP and dIMP from dITP. In contrast, E. coli extract with His-mNUDT16 generated only dIMP from dITP (Supplementary Figure S3). Thus, we concluded that mouse NUDT16 also hydrolyzes dIDP to dIMP.

Figure 2. His-NUDT16 hydrolyzes (d)IDP in preference to other nucleotides. (A) Samples from the purification of recombinant His-NUDT16 were subjected to SDS-PAGE and GelCode Blue Staining. Lane 1, supernatant of E. coli extract; lane 2, flow-through fraction from the His-tag purification; lane 3, eluate from His-tag purification; lane 4, fraction recovered by ammonium sulfate precipitation; lane 5, sample after dialysis; lane 6, eluate from cation exchange chromatography; lane 7, fraction recovered from gel filtration chromatography. (B) IDP (200 μM) was incubated with 400 nM His-NUDT16 for 1 h at 37°C. Reaction products were analyzed by HPLC (lower panel), and were compared with substrate IDP incubated without His-NUDT16 (upper panel). HPLC chromatograms of both samples obtained by absorbance at 249 nm are shown. (C) Nucleoside di- or triphosphates (10 or 100 μM) were incubated with 50 nM His-NUDT16 for 1 h at 37°C. The reaction products were analyzed by HPLC. The graph shows the concentration of each nucleoside monophosphate product.
Furthermore, most of the NUDT16 signals disappeared in cells treated with NUDT16 siRNA#1 (Figure 3C-h). We thus concluded that NUDT16 protein is mostly localized in nucleoli.

Knockdown of NUDT16 expression suppresses proliferation of HeLa MR cells

To elucidate the biological functions of NUDT16, we examined the effects of NUDT16 knockdown in HeLa MR cells. Twenty-four hours after the introduction of siRNA, the cells were reseeded. We then compared cell proliferation rates between cells treated with control siRNA#1 and those treated with NUDT16 siRNA#1 (Figure 4A). NUDT16 siRNA#1 significantly suppressed cell proliferation compared with the control siRNA (Figure 4B). Introduction of NUDT16 siRNA#2, which has a different target sequence for NUDT16, also similarly suppressed the proliferation of HeLa MR cells, confirming the effect of NUDT16 knockdown (Supplementary Figure S5A and B). Next, we performed a flow cytometric analysis of DNA content of HeLa MR cells after NUDT16 knockdown. Cell cycle analysis revealed significantly increased S-phase and decreased G1 phase populations after introduction of NUDT16 siRNA#1 (Figure 4C). Moreover, Hoechst 33342/PI
staining of the cells revealed that introduction of NUDT16 siRNA#1 caused no obvious increase in the dead cell fraction (PI positive) in comparison to control siRNA#1 (Supplementary Figure S6). We observed no subG1 fraction, indicating that NUDT16 knockdown did not induce cell death in our experimental conditions (Figure 4C).

Accumulation of inosine nucleotides in RNA and of single strand breaks in DNA after knockdown of NUDT16 expression

The localization of NUDT16 in nuclei strongly suggested that NUDT16 contributes to sanitization of the nuclear nucleotide pool, which supplies precursors for the synthesis of RNA and DNA. We, therefore, measured inosine in cellular RNA and deoxynosine in nuclear DNA in HeLa MR cells after NUDT16 knockdown by siRNA. The inosine level in RNA was significantly increased in cells treated with NUDT16 siRNA#1 (36.1 ± 1.35 inosine residues per 10^6 guanosine residues) compared with cells treated with control siRNA#1 (31.9 ± 1.05 inosine residues per 10^6 guanosine residues) (P = 0.0125) (Figure 5A right). However, there was no significant difference in the deoxynosine level in DNA between the two conditions (Figure 5A left). These results suggested that ITP or dITP levels are increased after NUDT16 knockdown, and that their incorporation into RNA or DNA may be similarly increased; however, deoxynosine incorporated into newly synthesized DNA might be quickly eliminated by DNA repair enzymes. DNA repair processes often form ssDNA regions as repair intermediates (30–32). Therefore, we examined ssDNA accumulation in nuclear DNA of HeLa MR cells by immunofluorescence detection using an anti-ssDNA antibody after siRNA treatment. The anti-ssDNA antibody was raised against fragmented bovine DNA, and recognizes ssDNA generated by double- or single-strand DNA breaks (33). Following treatment with NUDT16 siRNA, the percentage of ssDNA-positive HeLa MR cells (13.9%) was 5.3 times higher than that treated with control siRNA (P = 0.000252) (Figure 5B). ssDNA regions can be generated by either single- or double-strand breaks in DNA. In the comet assay, HeLa

![Graphs and images](https://academic.oup.com/nar/article-abstract/38/14/4834/2409624)

**Figure 5.** Knockdown of NUDT16 in HeLa MR cells increases the number of inosine residues in RNA and the level of single strand breaks in nuclear DNA. HeLa MR cells were independently transfected with siRNAs three times. Three days after transfection, cells were subjected to the following analysis. (A) Quantification of inosine or deoxynosine by LC-MS/MS. HeLa MR cells were harvested to determine the levels of inosine and deoxynosine [dI]. The numbers of deoxynosine residues [dI] per 10^6 nucleosides in DNA or inosine residues per 10^6 guanosine [G] in RNA from three independent transfections are shown. Student’s t-test, P = 0.0125 (RNA). (B) Knockdown of NUDT16 induces the accumulation of ssDNA in nuclei of HeLa MR cells. HeLa MR cells transfected with control siRNA#1 (a–c) or with NUDT16 siRNA#1 (d–f) were subjected to immunofluorescence microscopy with anti-ssDNA (green, b and e). Nuclei were stained with DAPI (blue, a and d). Merged signals are shown in c and f (blue and green). Percentages of ssDNA-positive nuclei among DAPI-positive nuclei are shown in the bar graph. Data are mean ± SD of three independent siRNA transfections. Student’s t-test, P = 0.000252. (C) Comet assay under alkaline conditions. Tail moments of at least 15 cells were calculated for each group and box-and-whisker plots are shown for three independent assays. Mann–Whitney U-test, P < 0.05. (D) Chromosomal abnormality. Transfected cells were prepared as in (B). Mitotic cells with chromosomal abnormalities (a) were defined as cells with chromatid breakage (b; solid arrowheads), chromatid gap (c; open arrowheads) and/or premature separation (d; arrows). These cells were counted and percentages of cells with chromosomal abnormalities among thirty mitotic cells are shown in the bar graph. Chromatid breakage, chromatid gap, and premature separation in control cells were 8%, 2% and 0%, respectively, and in NUDT16 knockdown cells were 12%, 4% and 1%, respectively. Data are mean ± SD from three independent siRNA transfections.
MR cells transfected with NUDT16 siRNA showed a significantly increased tail moment under alkaline conditions in three independent experiments (P < 0.05, Mann–Whitney U-test), but not under neutral conditions (Figure 5C and Supplementary Figure S7). Cells exposed to hydrogen peroxide, which is known to cause double-strand breaks, exhibited a significantly increased tail moment, even under neutral conditions; therefore we concluded that knockdown of NUDT16 caused accumulation of single-strand breaks in nuclear DNA. Next, chromosome abnormalities including chromatid breakage, chromatid gap, and/or premature separation were examined in mitotic cells. The percentage of the cells with abnormal chromosomes in HeLa MR cells treated with NUDT16 siRNA was 1.8 times higher than that in cells treated with control siRNA, although the difference was not significant (P = 0.115) (Figure 5D).

**DISCUSSION**

In the present study, we reported two major findings; first, NUDT16 hydrolyzes (d)IDP/(d)ITP and second, NUDT16 deficiency induces accumulation of single strand breaks in nuclear DNA and growth arrest in human cells.

Previously, Ghosh et al. (34) reported that NUDT16 recognizes the 5'-cap structure of U8 small nucleolar RNA (snRNA) and weakly hydrolyzes it to produce non-capped-snoRNA with guanosine 5'-monophosphate at its 5'-terminus and an excited cap. First guanosine residue of U8 snoRNA itself is linked to the triphosphate following the 5'-cap, and thus mimicking a GTP structure. GTP was shown to be weakly hydrolyzed by NUDT16 in the present study. Therefore, both 5'-capped U8 snoRNA and GTP can be converted by NUDT16 to guanosine 5'-monophosphate (GMP) at the 5'-terminal end of snoRNA and to free GMP, respectively. Thus, it is likely that hydrolysis of GTP and decapping of U8 snoRNA are essentially the same enzyme reaction of NUDT16. We also showed that in a human cell line, NUDT16 is localized in nuclei, mainly nucleoli. Similarly, Ghosh et al. (34) have reported that X29 protein, the Xenopus homolog of NUDT16, is primarily a nucleolar protein in Xenopus cells in vitro.

As shown in Figure 6, NUDT16 efficiently hydrolyzes (d)IDP, and hydrolyzes (d)ITP to a lesser extent. (d)ITP and (d)IDP can be generated by deamination of adenine nucleotides or phosphorylation of (d)IMP (35). ITPA, which is relatively abundant in the cytoplasm and which is encoded by ITPA, is known to hydrolyze (d)ITP to (d)IMP and pyrophosphate (14,15). The study of NUDT16 reaction kinetics revealed that NUDT16 has a lower hydrolysis rate but a higher affinity for (d)ITP compared with ITPA (for ITP, NUDT16, kat 3.2 min⁻¹, Km 22.1 μM; ITPA, kat 34 800 min⁻¹, Km 510 μM) (15). NUDT16, therefore, might be an important enzyme for the elimination of (deoxy)inosine nucleotides from nuclei, especially at low concentrations. Because NUDT16 is localized mainly in nuclei, NUDT16 may prevent the incorporation of inosine nucleotides into ribosomal RNA (rRNA) during transcription. An increase in the RNA inosine level, observed after the knockdown of NUDT16 supports this hypothesis. NUDT16, but not ITPA, has a strong hydrolysis activity for (d)IDP. In the pathway converting abundant cellular IMP to ITP by phosphorylation (10), IDP is an important intermediate. In addition, IDP is expected to be converted to dIDP by ribonucleotide reductase (36), and dIDP can be phosphorylated, thereby resulting in increased levels of dITP (11).

8-oxo-dGTP is known to be incorporated into genomic DNA and to induce mutation in both mammalian and bacterial cells (3). Human NUDT5, which hydrolyzes 8-oxo-dGDP, was reported to decrease spontaneous mutation in E. coli mutT⁻ cells deficient in 8-oxo-dGTP hydrolyzing activity (37). Taken together with our findings on NUDT16, these results support the importance of the elimination of modified (deoxy)nucleoside diphosphates from the nucleotide pools.

In the present study, we observed the deoxyinosine levels in nuclear DNA within the normal ranges previously observed by Taghizadeh et al. (27) even after knockdown of NUDT16. However, knockdown of NUDT16 expression increased the fraction of ssDNA-positive cells. We assume that the dITP level in the nuclear nucleotide pool must increase after NUDT16 knockdown, thus resulting in increased incorporation of deoxyinosine into newly synthesized DNA. The deoxyinosine in DNA is immediately removed by the DNA repair process, resulting in an accumulation of ssDNA. Bradshaw and Kuzminov (38) described the incorporation of dITP/dXTP into the genomic DNA of rdgB⁻ E. coli cells, which lack bacterial ITPase. In E. coli cells, DNA containing deoxyinosine can be excised by Endo V-initiated nucleotide excision repair, thus resulting in DNA strand breakage (32). Both Endo V and another enzyme, alkyl-adenine-DNA glycosylase (AAG, MAG, ANPG, MPG) were reported as candidates for hypoxanthine specific DNA repair enzymes in
mammalian cells (39–41). It is well known that increased accumulation of ssDNA triggers the DNA damage response, to induce delay in S-phase, and then cell cycle arrest (42). We, therefore, assume that the DNA damage response, to the accumulation of ssDNA, suppresses cell cycle progression, thus increasing the S-phase population and resulting in a decreased proliferation rate. On the other hand, knockdown of NUDT16 expression induced a 13.3% increase in RNA inosine levels. RNA editing by adenosine deaminases is a well-known system of post-transcriptional regulation and the major source of inosine in RNA; therefore, more inosine residues are present in RNA compared with deoxyinosine residues in DNA (43,44). Inosine, produced in RNA by such a regulated system, is thought to cause important modifications to the functions of non-coding RNA, or to alter amino acid sequence encoded by mRNA (45,46). In other words, unregulated incorporation of inosine during RNA transcription might impair RNA functions. In the present study, we demonstrated that NUDT16 contributes to the suppression of such inosine incorporation into RNA. Although the increased level of inosine in RNA under NUDT16 deficiency was statistically significant and the net increase was much higher than the basal level of deoxyinosine in DNA (4.3 inosine/10^6 G versus 0.63 deoxyinosine/10^6 dG), the higher basal level of inosine in RNA (32 inosine/10^6 G), which is likely to be generated by RNA-editing (45,46), made the difference appear small.

We previously reported that Itpa–/– mice showed growth retardation and heart failure and did not survive beyond 2 weeks after birth (16). Following this report, we found that primary mouse embryonic fibroblasts (MEFs) prepared from Itpa–/– mice showed a significantly prolonged doubling time and chromosomal abnormalities, accompanied by increased ssDNA and deoxyinosine residues in nuclear DNA (47). However, once Itpa–/– MEFs were spontaneously immortalized, the immortalized Itpa–/– MEFs had neither of these phenotypes. Furthermore, immortalized Itpa–/– MEFs exhibited significantly increased levels of Nudt16 mRNA and protein. siRNA-mediated knockdown of Nudt16 in immortalized Itpa–/– MEFs significantly increased deoxyinosine levels in nuclear DNA, and thus reproduced the ITPA-deficient phenotype. We, therefore, concluded that mouse NUDT16 functions as a backup enzyme for the ITPA deficiency by eliminating (d)IDP, and to a lesser extent (d)ITP from the nucleotide pools in MEFs. In wild-type MEFs, knockdown of Nudt16 did not result in such phenotypes, indicating that mouse ITPA can compensate for the deficiency of NUDT16 in MEFs. In contrast, our present data suggested that human ITPA itself cannot completely compensate for the deficiency of NUDT16 in HeLa MR cells, although double deficiency of ITPA and NUDT16 would cause more severe phenotypes. Human individuals with ITPA deficiency show no obvious phenotypes. Thus, ITPA deficiency causes quite different effects in mouse and human. Comparing the expression level or enzyme activity of NUDT16 between mouse and human might explain why human cells have a significant tolerance to ITPA deficiency. In the present study, partial reduction of NUDT16 expression in HeLa MR cells was sufficient to cause growth suppression and accumulation of single-strand breaks in nuclear DNA. Therefore, defective NUDT16 might lead to genome-instability syndromes in human individuals.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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