



Title	Suppression of mutagenesis by 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-triphosphate) by human MTH1, MTH2, and NUDT5
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**Suppression of mutagenesis by 8-hydroxy-2'-deoxyguanosine  
5'-triphosphate (7,8-dihydro-8-oxo-2'-deoxyguanosine  
5'-triphosphate) by human MTH1, MTH2, and NUDT5**

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**Abstract**

To assess the functions of the three human MutT-type enzymes, MTH1, MTH2, and NUDT5, mutation induction by an oxidized form of dGTP, 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP, 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-triphosphate), was examined using human 293T cells treated with their specific siRNAs. Shuttle plasmid DNA containing the *supF* gene was first transfected into the cells, and then 8-OH-dGTP was introduced by means of osmotic pressure. *Escherichia coli* cells were transformed with the DNAs replicated in the treated cells. The knock-downs of the MTH1, MTH2, and NUDT5 proteins increased the A:T→C:G substitution mutations induced by 8-OH-dGTP. In addition, the increase in the induced mutation frequency was more evident in the triple knock-down cells. These results indicate that all three of the human MTH1, MTH2, and NUDT5 proteins act as a defense against the mutagenesis induced by oxidized dGTP.

Normal cellular metabolism produces endogenous reactive oxygen species (ROS). ROS are generated as byproducts of the mitochondrial electron transport chain, and certain cellular enzymes also generate ROS. Moreover, ROS are produced by environmental mutagens/carcinogens, including ionizing radiation and ultraviolet light. The formation of ROS leads to the oxidation of cellular components and disturbs their normal functions. The formation of oxidized DNA lesions is one of the causative factors of mutagenesis, carcinogenesis, neurodegeneration, and aging [1-5].

DNA precursors (2'-deoxyribonucleotides) are also subjected to oxidative damage. The formation of oxidized DNA precursors is a potential source of mutagenesis [6]. 8-Hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP, 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-triphosphate) is the major oxidation product of dGTP in *in vitro* oxidation reactions [7]. 8-OH-dGTP was reportedly present at a concentration of 1–10% relative to the unmodified dGTP in the mitochondrial nucleotide pool [8]. This oxidized form of dGTP is highly mutagenic in living cells when added exogenously [9-11] and is expected to act as an endogenous mutagen.

Nucleotide pool sanitization is an important means by which organisms prevent the mutagenesis caused by damaged DNA precursors [6,12]. The *Escherichia coli* MutT (NudA) protein, encoded by the mutator gene *mutT*, was the first enzyme found to degrade an oxidized DNA precursor (8-OH-dGTP) *in vitro* [13]. Moreover, at least two *E. coli*

MutT-type proteins, Orf135 (NudG) and Orf17 (NudB), in addition to MutT, catalyze the hydrolysis of oxidized DNA precursors *in vitro* [14,15]. *E. coli* strains lacking MutT and Orf135 exhibit a mutator phenotype [16-19], indicating the importance of nucleotide pool sanitization to prevent mutagenesis by oxidized DNA precursors. Mammalian cells also possess MutT-type enzymes. The MTH1 (NUDT1) protein catalyzes the hydrolysis of various oxidized DNA precursors, including 8-OH-dGTP, and greater numbers of tumors were formed in the lungs, livers, and stomachs of MTH1-deficient mice than wild-type mice [20,21]. MTH2 (NUDT15) degrades 8-OH-dGTP *in vitro*, and the expression of the cDNA encoding MTH2 significantly reduced the elevated spontaneous mutation frequency in *E. coli mutT* cells [22]. The NUDT5 protein is unique, because the hydrolysis of 8-hydroxy-2'-deoxyguanosine 5'-diphosphate (8-OH-dGDP, 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-diphosphate) by this protein is much more efficient than that of 8-OH-dGTP [23,24]. However, the expression of NUDT5 in *mutT*-deficient *E. coli* mutant cells decreases the spontaneous mutation frequency to the normal level, suggesting the importance of its 8-OH-dGDPase activity in nucleotide pool sanitization [23]. Thus, it is quite important to examine whether the three proteins prevent the mutations induced by 8-OH-dGTP in mammalian (human) cells.

Recently, we showed that 8-OH-dGTP specifically induced A:T→C:G transversions in live simian and human cells [10,11]. In this study, to assess the contributions of these MutT-type proteins to the prevention of the mutagenesis caused by 8-OH-dGTP in human cells, we suppressed their expression by siRNAs and introduced 8-OH-dGTP into the knocked-down cells. The knock-downs of all of the MutT-type proteins enhanced the A:T→C:G substitution mutations, suggesting their roles as functional nucleotide pool sanitization enzymes.

## **Materials and methods**

### *Materials*

8-OH-dGTP was purchased from TriLink BioTechnologies (San Diego, California, USA). dGTP (FPLC-grade) was obtained from GE Healthcare Bio-Sciences (Piscataway, New Jersey, USA). Purified oligonucleotides were from Sigma Genosys Japan (Ishikari, Japan). siRNAs (“stealth RNAi”; Invitrogen, Carlsbad, California, USA) were synthesized according to the BLOCK-iT RNAi Designer software, on the supplier’s Web site. The following siRNAs were used: MTH1 sense, 5'-AUCUGAUCCAGCUGGAACCAGCAUG; MTH1 antisense, 5'-CAUGCUGGUUCCAGCUGGAUCAGAU; MTH2 sense,

5'-UUCCCAAGGAACCCACUCCCAACUU; MTH2 antisense,  
 5'-AAGUUGGGAGUGGGUCCUUGGGAA; NUDT5 sense,  
 5'-AGAAGAUCCGUUGGUUCUUGGCUC; NUDT5 antisense,  
 5'-GAGCCAAGAACCAACGGAAUCUUCU. Negative Control Medium  
 GC Duplex was used as a control siRNA (Invitrogen). The pZ189-StuI  
 plasmid was constructed in our laboratory [11]. The *E. coli* strain  
 KS40/pOF105 [25] was provided by Dr. Tatsuo Nunoshiba, of  
 International Christian University, and was used as an indicator strain of  
 the *supF* mutants.

#### *Mutagenesis experiments*

293T cells ( $3 \times 10^4$  cells) were plated into 24-well dishes and were  
 cultured in Dulbecco's modified Eagle medium supplemented with 10%  
 fetal calf serum, at 37°C under a 5% CO<sub>2</sub> atmosphere for 24 hr. siRNAs  
 (7.2 pmol each) were mixed with Lipofectamine (Invitrogen) and  
 introduced into the cultured 293T cells according to the supplier's  
 recommendations. In the triple knock-down experiment, an siRNA cocktail  
 (MTH1; 3.6 pmol, MTH2; 7.2 pmol and NUDT5; 3.6 pmol) was used.  
 After 24 hr, the pZ189-StuI plasmid (58 fmol, 200 ng) was mixed with  
 Polyfect (Qiagen, Hilden, Germany) and transfected into the cultured 293T  
 cells according to the supplier's recommendations. After 24 hr,  
 8-OH-dGTP (2.4 pmol) was introduced by osmotic pressure, using a buffer

solution (30 mM KCl, 10 mM Hepes–NaOH, pH 7.4), according to the literature [26]. After 24 hr of culture, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin [27]. The recovered DNA was treated with DpnI, to digest the unreplicated plasmids.

The plasmids recovered from the 293T cells were transfected into *E. coli* KS40/pOF105 cells by electroporation, using a Gene Pulser II Transfection Apparatus with a Pulse Controller II (Bio-Rad, Hercules, California, USA). The *supF* mutant frequency was calculated according to the numbers of white and pale blue colonies on Luria–Bertani agar plates containing nalidixic acid (50  $\mu\text{g/ml}$ ), streptomycin (100  $\mu\text{g/ml}$ ), ampicillin (150  $\mu\text{g/ml}$ ), chloramphenicol (30  $\mu\text{g/ml}$ ), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (80  $\mu\text{g/ml}$ ), and isopropyl- $\beta$ -D-thiogalactopyranoside (23.8  $\mu\text{g/ml}$ ), and the numbers of colonies on agar plates containing ampicillin and chloramphenicol, as described in Ref. [25].

#### *Quantitative RT-PCR analysis of mRNA*

Total RNA was extracted from 293T cells using an RNeasy Mini Kit (Qiagen) combined with RNase-free DNase I (Takara, Otsu, Japan), for the degradation of the genomic DNA in the total RNA samples. First-strand cDNA synthesis was performed with 500 ng of total RNA using an oligo dT primer and an RNA PCR Kit (AMV) (Takara), according to the manufacturer's instructions. Each of the mRNA transcripts was measured



by a quantitative PCR method with an ABI 7500 Real Time PCR System and SYBR-Green chemistry (Applied Biosystems, Foster City, CA) using the following primer sets:

	MTH1	upper,
5'-dAGGAGGAGAGCGGTCTGACA;	MTH1	lower,
5'-dCACGAACTCAAACACGATCTG;	MTH2	upper,
5'-dGAAAGGAGAAGTGGATGTGAC;	MTH2	lower,
5'-dGGAACCCACTCCCAACTTTC;	NUDT5	upper,
5'-dGTTCTCCAGCGGTCTGTATG;	NUDT5	lower,

5'-dCTTCGGCCTTGCGTTTTTCG. Data are expressed as the ratio to the GAPDH mRNA, which was determined using the following primers: GAPDH upper, 5'-dAACTTTGGTATCGTGGAAGG; GAPDH lower, 5'-dGTCTTCTGGGTGGCAGTGAT.

### *Statistical analysis*

Statistical significance was examined by the Student's *t*-test. Levels of  $P < 0.05$  were considered to be significant.

## **Results**

### *Knock-downs of MutT-type enzymes enhanced the induced mutations*

Recently, we introduced 8-OH-dGTP into human cells and found that it specifically induced A:T→C:G transversion mutations [11]. We first examined the effects of the single knock-down of MTH1, MTH2, and NUDT5 by siRNAs. These knock-downs were measured by quantitative RT (reverse transcription)-PCR (Table 1). The treatment with siRNA reduced the amounts of mRNAs encoding these MutT-type proteins.

When unmodified dGTP was introduced by means of osmotic pressure, the observed *supF* mutant frequency was  $\sim 1 \times 10^{-4}$  in the cells treated with the control siRNA (Fig. 1). The mutant frequencies upon the treatment with siRNAs against MTH1, MTH2, and NUDT5 were similar to that of the control cells, indicating that the reduced amounts of these proteins did not affect the background mutant frequency in this experimental system. The introduction of 8-OH-dGTP enhanced the total mutant frequency to  $\sim 4 \times 10^{-4}$  in the cells treated with the control siRNA. We then introduced 8-OH-dGTP into the knocked-down cells. The *supF* mutant frequencies were affected by the knock-down of all three of the MutT-type enzymes examined in this study. As shown in Fig. 1, the induced mutant frequencies were significantly increased ( $\sim 6 \times 10^{-4}$ ) in the cells in which the expression of MTH1, MTH2, and NUDT5 was suppressed. Thus, the MTH1 and MTH2 proteins, which have 8-OH-dGTPase activity, were shown to prevent the mutagenesis caused by 8-OH-dGTP. In addition, the results from the NUDT5-knock-down

experiment suggested that the endogenous NUDT5 protein with the 8-OH-dGDPase activity also suppresses the mutagenesis by the triphosphate derivative 8-OH-dGTP.

The mutation spectra in the knocked-down cells are shown in Tables 2 and 3. In all cells treated with siRNAs, 8-OH-dGTP specifically induced A:T→C:G transversions. In contrast, A:T→C:G mutations were infrequently found in the dGTP experiments. These results indicate that the knock-down of these MutT-type enzymes enhanced the induced A:T→C:G mutations, supporting the interpretation that these proteins act as a defense against 8-OH-dGTP.

The overall distributions of the mutations were unchanged by the knock-downs, as shown in Supplementary Tables 1 and 2.

#### *Effects of the triple knock-down of MutT-type enzymes*

We next examined the effects of the triple knock-down of MTH1, MTH2, and NUDT5. These knock-down was measured by quantitative RT-PCR (Table 4). Lower amounts of siRNAs were used for MTH1 and NUDT5 in the triple knock-down experiment, because of toxicity, and the efficiency of the mRNA reduction was lower than those in the single knock-down experiments. Again, the triple knock-down of these proteins did not affect the background mutant frequency in this experimental system ( $\sim 1 \times 10^{-4}$ , Fig. 2).

The triple knock-down enhanced the mutant frequency induced by 8-OH-dGTP ( $\sim 4$  to  $\sim 7 \times 10^{-4}$ , Fig. 2). The enhancement was more evident for the triple knock-down than for the single knock-downs (Figs. 1 and 2), although the mRNAs of each MutT-type enzyme were more abundant in the former case than in the latter (Tables 1 and 4). Again, 8-OH-dGTP specifically induced A:T→C:G transversions (Table 5). The overall distributions of the mutations are shown in Supplementary Table 3. These results suggest that the three MutT-type enzymes exhibit mutually complementary roles in the elimination of 8-OH-dGTP from the nucleotide pool.

## Discussion

The major objective of this study was to examine whether the human MutT-type enzymes, MTH1, MTH2, and NUDT5, actually function as nucleotide pool sanitization enzymes in living cells. These proteins were knocked-down in human cells, and 8-OH-dGTP was introduced into the knocked-down cells. As shown in Fig. 1 and Table 3, the reduction in these proteins significantly increased the 8-OH-dGTP-induced mutations. The MTH1 and MTH2 proteins catalyze the hydrolysis of 8-OH-dGTP *in vitro* [20,22], and the results shown in Fig. 1 are in agreement with the *in vitro* observations. The 8-OH-dGTPase activities of MTH1 and MTH2 play

important roles in the suppression of the mutagenesis induced by 8-OH-dGTP in living human cells. Because the effects of their single knock-down were similar (at the mRNA level), the contributions of the two proteins seem to be comparable in this human cell line.

In addition, the knock-down of NUDT5 enhanced the mutagenicity of 8-OH-dGTP (Fig. 1 and Table 3). This unique MutT-type protein catalyzes the hydrolysis of 8-OH-dGDP, rather than 8-OH-dGTP [23,24]. Since 8-OH-dGTP and 8-OH-dGDP are interconvertible within a cell [28], the decreased 8-OH-dGDPase activity in the NUDT5-knocked-down cells could increase the 8-OH-dGTP concentration. Moreover, Ishibashi *et al.* proposed [23] that the hydrolysis of 8-OH-dGDP, a potent inhibitor of the MTH1 protein [29,30], could promote the function of MTH1. It is not known whether 8-OH-dGDP is another substrate or an inhibitor for MTH2. If it is an inhibitor, then the knock-down of NUDT5 could suppress the 8-OH-dGTPase activities of both MTH1 and MTH2, resulting in enhanced mutation frequencies induced by 8-OH-dGTP. However, in contrast to this explanation, the expression of NUDT5 in *mutT*-deficient *E. coli* mutant cells decreases the spontaneous mutation frequency to the normal level [23], although the *E. coli* MutT protein catalyzes the hydrolyses of both 8-OH-dGTP and 8-OH-dGDP [31]. Thus, the decreased mutation frequency (the 8-OH-dGTP concentration) could not be explained by the degradation of the 8-OH-dGTPase inhibitor, in the case of NUDT5

expressed in bacterial cells. Although the biological significance of the unique activity of NUDT5 is still open to debate, its contribution to the nucleotide pool sanitization has been confirmed in this study (Fig. 1 and Table 3).

Recently, Mundt *et al.* reported that extracellular  $^{14}\text{C}$ -labeled 8-hydroxy-2'-deoxyguanosine was converted to nucleoside 5'-triphosphates in human cells [32]. They demonstrated that the radiocarbon content of DNA and RNA was decreased by Immucillin H (an inhibitor of purine nucleoside phosphorylase) and that hydroxyurea (an inhibitor of ribonucleotide reductase (RR)) suppressed the radiocarbon incorporation into DNA, but not into RNA. They proposed that 8-hydroxyguanosine 5'-monophosphate and subsequently 5'-diphosphate were synthesized from 8-hydroxyguanine (free base) that was the phosphorolysis product of 8-hydroxy-2'-deoxyguanosine by the function of purine nucleoside phosphorylase and that 8-hydroxyguanosine 5'-diphosphate was converted to 8-OH-dGDP by RR. Based on this report, oxidation of dG, guanine, GMP and GDP, in addition to that of dGMP, dGDP and dGTP, could be an important source of 8-OH-dGTP in cells. Given that NUDT5 catalyzes the hydrolysis of 8-OH-dGDP and its ribonucleotide counterpart [33], this activity would be important for the suppression of intracellular 8-OH-dGTP concentration, although we consider only the 8-OH-dGDPase activity in this study because we used exogenous 8-OH-dGTP. However, this

interpretation should be reviewed carefully, since Hayakawa *et al.* reported that RR did not convert 8-hydroxyguanosine 5'-diphosphate to 8-OH-dGDP [34].

Tsuzuki *et al.* found that a greater number of tumors were spontaneously formed in the lungs, livers, and stomachs of MTH1-deficient mice, as compared with wildtype mice [21]. Thus, it would be interesting to examine the phenotypes of animals deficient in MTH2 and NUDT5. Based on the results shown in Fig. 1, tumors could form in the MTH2 and NUDT5 knock-out animals with higher frequencies than in the control animals. Moreover, double- and triple-knock-out animals of the mammalian MutT-type enzymes might exhibit more prominent phenotypes. Analyses of these animals would reveal the roles of the MutT-type enzymes in the suppression of tumor formation.

2-Hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP), another substrate for the MTH1 protein [30,35], synergistically enhanced the mutagenicity of 8-OH-dGTP in *in vitro* DNA replication with a human cell extract [36]. 2-OH-dATP suppressed the hydrolysis of 8-OH-dGTP in the extract, suggesting that the inhibition of the MTH1-like protein(s) plays major roles in the enhancement of the mutagenicity. These results highlight the importance of the specific hydrolysis of 8-OH-dGTP for the suppression of its induced mutation. Because the knock-downs of MTH1, MTH2, and NUDT5 enhanced the induced mutations in living human cells

(Fig. 1), all of these MutT-type proteins could reduce the amount of 8-OH-dGTP present in the extract. Thus, 2-OH-dATP might inhibit the activities of these enzymes in the extract. In agreement with this speculation, 2-hydroxy-2'-deoxyadenosine 5'-diphosphate is a substrate for NUDT5 [24]. The hydrolyzing activity of MTH2 for 2-OH-dATP has not been reported, and further studies are necessary to address this possibility.

Nakabeppu and his colleagues reported that both the 8-OH-dGTPase and 2-OH-dATPase activities of MTH1 contributed to the suppression of H<sub>2</sub>O<sub>2</sub>-induced mitochondrial dysfunction and cell death [37]. In addition, Rai *et al.* recently reported that the suppression of MTH1 expression in cells caused senescence [38]. The ability of the MTH2 and NUDT5 proteins to suppress the mutations induced by 8-OH-dGTP (Fig. 1) suggests that they also could contribute to the suppression of ROS-induced cell death. Furthermore, the decreased expression of MTH1 in the CA3 subregion of the brain in Alzheimer's disease subjects [39] and the progressive decrease in MTH2 in the hippocampus of the senescence-accelerated prone mouse 8 (SAMP8) [40] suggest the possibility that the three mammalian MutT-type enzymes contribute to the suppression of neurodegeneration and aging.

Recently, a physical interaction between MTH2 and PCNA was reported [41]. The knock-down of MTH2 significantly promoted the degradation of PCNA, and UV irradiation accelerated PCNA degradation



by inducing the dissociation of PCNA-MTH2. Moreover, the knock-down of MTH2 inhibited DNA synthesis and enhanced cell cycle progression. However, judging by the numbers of colonies on the titer plates, which semi-quantitatively reflected the amounts of plasmid DNA replicated in the cells, no obvious effect of the MTH2 knock-down was observed (data not shown). However, the numbers of *E. coli* colonies are affected by the electroporation efficiency, and further studies will be required to clarify the effects of the MTH2 knock-down.

*E. coli* cells contain three MutT-type proteins (MutT, Orf135, and Orf17) that catalyze the hydrolysis of 8-OH-dGTP *in vitro* [13-15]. When 8-OH-dGTP was introduced into *E. coli* strains lacking MutT or Orf135, and a strain expressing the antisense RNA for Orf17, a phenotype was observed only for *mutT* cells [18,19,42]. Thus, MutT is the major enzyme for specific 8-OH-dGTP degradation in *E. coli*, and Orf135 and Orf17 back up MutT. In contrast, the three MutT-type proteins act to similar degrees in human cells, at least in 293T cells. This could be the reason for the weak mutator phenotype of mouse cell lines deficient in MTH1 [43].

In conclusion, this study demonstrated that the knock-downs of MTH1, MTH2, and NUDT5 increased the mutagenicity of 8-OH-dGTP in human 293T cells. This result suggests that the three MutT-type proteins act as a primary defense against the mutagenesis induced by oxidized dGTP.

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**REFERENCES**

- [1] Halliwell, B.; Gutteridge, J.M. Free Radicals in Biology and Medicine, second ed. Clarendon: Oxford; 1989.
- [2] Ames, B.N. Dietary carcinogens and anticarcinogens. *Science* **221**:1256-1264; 1983.
- [3] Harman, D. The aging process, *Proc. Natl. Acad. Sci. U.S.A.* **78**:7124-7128; 1981.
- [4] Kamiya, H. Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides. *Nucleic Acids Res.* **31**:517-531; 2003.
- [5] Hiraku, Y.; Murata, M.; Kawanishi, S. Role of oxidative DNA damage in dietary carcinogenesis. *Genes Environ.* **28**:127-140; 2006.
- [6] Kamiya, H. Mutations induced by oxidized DNA precursors and their prevention by nucleotide pool sanitization enzymes. *Genes Environ.* **29**:133-140; 2007.
- [7] Murata-Kamiya, N.; Kamiya, H.; Muraoka, M.; Kaji, H.; Kasai, H. Comparison of oxidation products from DNA components by  $\gamma$ -irradiation and Fenton-type reactions. *J. Radiat. Res.* **38**:121-131; 1997.

- [8] Pursell, Z.F.; McDonald, J.T.; Mathews, C.K.; Kunkel, T.A. Trace amounts of 8-oxo-dGTP in mitochondrial dNTP pools reduce DNA polymerase  $\gamma$  replication fidelity. *Nucleic Acids Res.* **36**:2174-2181; 2008.
- [9] Inoue, M.; Kamiya, H.; Fujikawa, K.; Ootsuyama, Y.; Murata-Kamiya, N.; Osaki, T.; Yasumoto, K.; Kasai, H. Induction of chromosomal gene mutations in *Escherichia coli* by direct incorporation of oxidatively damaged nucleotides. *J. Biol. Chem.* **273**:11069-11074; 1998.
- [10] Satou, K.; Kawai, K.; Kasai, H.; Harashima, H.; Kamiya, H. Mutagenic effects of 8-hydroxy-dGTP in live mammalian cells. *Free Radic. Biol. Med.* **42**:1552-1560; 2007.
- [11] Satou, K.; Hori, M.; Kawai, K.; Kasai, H.; Harashima, H.; Kamiya, H. Involvement of specialized DNA polymerases in mutagenesis by 8-hydroxy-dGTP in human cells. *DNA Repair (Amsterdam)* **8**:637-642; 2009.
- [12] Sekiguchi, M.; Tsuzuki, T. Oxidative nucleotide damage: consequences and prevention. *Oncogene* **21**:8895-8904; 2002.
- [13] Maki, H.; Sekiguchi, M. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* **355**:273-275; 1992.

- [14] Kamiya, H.; Murata-Kamiya, N.; Iida, E.; Harashima, H. Hydrolysis of oxidized nucleotides by the *Escherichia coli* Orf135 protein. *Biochem. Biophys. Res. Commun.* **288**:499-502; 2001.
- [15] Hori, M.; Fujikawa, K.; Kasai, H.; Harashima, H.; Kamiya, H. Dual hydrolysis of diphosphate and triphosphate derivatives of oxidized deoxyadenosine by Orf17 (NtpA), a MutT-type enzyme. *DNA Repair (Amsterdam)* **4**:33-39; 2005.
- [16] Yanofsky, C.; Cox, E.C.; Horn, V. The unusual mutagenic specificity of an *E. coli* mutator gene. *Proc. Natl. Acad. Sci. U.S.A.* **55**:274-281; 1966.
- [17] Tajiri, T.; Maki, H.; Sekiguchi, M. Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat. Res.* **336**:257-267; 1995.
- [18] Kamiya, H.; Ishiguro, C.; Harashima, H. Increased A:T→C:G mutations in the *mutT* strain upon 8-hydroxy-dGTP treatment: direct evidence for MutT in the prevention of mutations by oxidized dGTP. *J. Biochem.* **136**:359-362; 2004.
- [19] Kamiya, H.; Iida, E.; Murata-Kamiya, N.; Yamamoto, Y.; Miki, T; Harashima, H. Suppression of spontaneous and hydrogen peroxide-induced mutations by a MutT-type nucleotide pool

- sanitization enzyme, the *Escherichia coli* Orf135 protein. *Genes Cells* **8**:941-950; 2003.
- [20] Mo, J.-Y.; Maki, H.; Sekiguchi, M. Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc. Natl. Acad. Sci. U.S.A.* **89**:11021-11025; 1992.
- [21] Tsuzuki, T.; Egashira, A.; Igarashi, H.; Iwakuma, T.; Nakatsuru, Y.; Tominaga, Y.; Kawate, H.; Nakao, K.; Nakamura, K.; Ide, F.; Kura, S.; Nakabeppu, Y.; Katsuki, M.; Ishikawa, T.; Sekiguchi, M. Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 11456-11461; 2001.
- [22] Cai, J.-P.; Ishibashi, T.; Takagi, Y.; Hayakawa, H.; Sekiguchi, M. Mouse MTH2 protein which prevents mutations caused by 8-oxoguanine nucleotides. *Biochem. Biophys. Res. Commun.* **305**:1073-1077; 2003.
- [23] Ishibashi, T.; Hayakawa, H.; Sekiguchi, M. A novel mechanism for preventing mutations caused by oxidation of guanine nucleotides. *EMBO Rep.* **4**:479-483; 2003.
- [24] Kamiya, H.; Hori, M.; Arimori, T.; Sekiguchi, M.; Yamagata, Y.; Harashima, H. NUDT5 hydrolyzes oxidized deoxyribonucleoside

- diphosphates with broad substrate specificity. *DNA Repair (Amsterdam)* **8**:1250-1254; 2009.
- [25] Obata, F.; Nunoshiba, T.; Hashimoto-Gotoh, T.; Yamamoto, K. An improved system for selection of forward mutations in an *Escherichia coli supF* gene carried by plasmids. *J. Radiat. Res.* **39**:263-270; 1998.
- [26] Koberna, K.; Staněk, D.; Malínský, J.; Eltsov, M.; Pliss, A.; Cvrtná, V.; Cermanová, S.; Rasvka, I. Nuclear organization studied with the help of a hypotonic shift: its use permits hydrophilic molecules to enter into living cells. *Chromosoma* **108**:325-335; 1999.
- [27] Stary, A.; Sarasin, A. Simian virus 40 (SV40) large T antigen-dependent amplification of an Epstein-Barr virus-SV40 hybrid shuttle vector integrated into the human HeLa cell genome. *J. Gen. Virol.* **73**:1679-1685; 1992.
- [28] Hayakawa, H.; Taketomi, A.; Sakumi, K.; Kuwano, M.; Sekiguchi, M. Generation and elimination of 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate, a mutagenic substrate for DNA synthesis, in human cells. *Biochemistry* **34**:89-95; 1995.
- [29] Bialkowski, K.; Kasprzak, K.S. A novel assay of 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation

- of cadmium(II) inhibition of this activity. *Nucleic Acids Res.* **26**:3194-3201; 1998.
- [30] Fujikawa, K.; Kamiya, H.; Yakushiji, H.; Fujii, Y.; Nakabeppu, Y.; Kasai, H. The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. *J. Biol. Chem.* **274**:18201-18205; 1999.
- [31] Ito, R.; Hayakawa, H.; Sekiguchi, M.; Ishibashi, T. Multiple enzyme activities of *Escherichia coli* MutT protein for sanitization of DNA and RNA precursor pools. *Biochemistry* **44**:6670-6674; 2005.
- [32] Mundt, J.M.; Hah, S.S.; Sumbad, R.A.; Schramm, V.; Henderson, P.T. Incorporation of extracellular 8-oxodG into DNA and RNA requires purine nucleoside phosphorylase in MCF-7 cells. *Nucleic Acids Res.* **36**:228-236; 2008.
- [33] Ishibashi, T.; Hayakawa, H.; Ito, R.; Miyazawa, M.; Yamagata, Y.; Sekiguchi, M. Mammalian enzymes for preventing transcriptional errors caused by oxidative damage. *Nucleic Acids Res.* **33**:3779-3784; 2005.
- [34] Hayakawa, H.; Hofer, A.; Thelander, L.; Kitajima, S.; Cai, Y.; Oshiro, S.; Yakushiji, H.; Nakabeppu, Y.; Kuwano, M.; Sekiguchi, M. Metabolic fate of oxidized guanine ribonucleotides in mammalian cells. *Biochemistry* **38**:3610-3614; 1999.



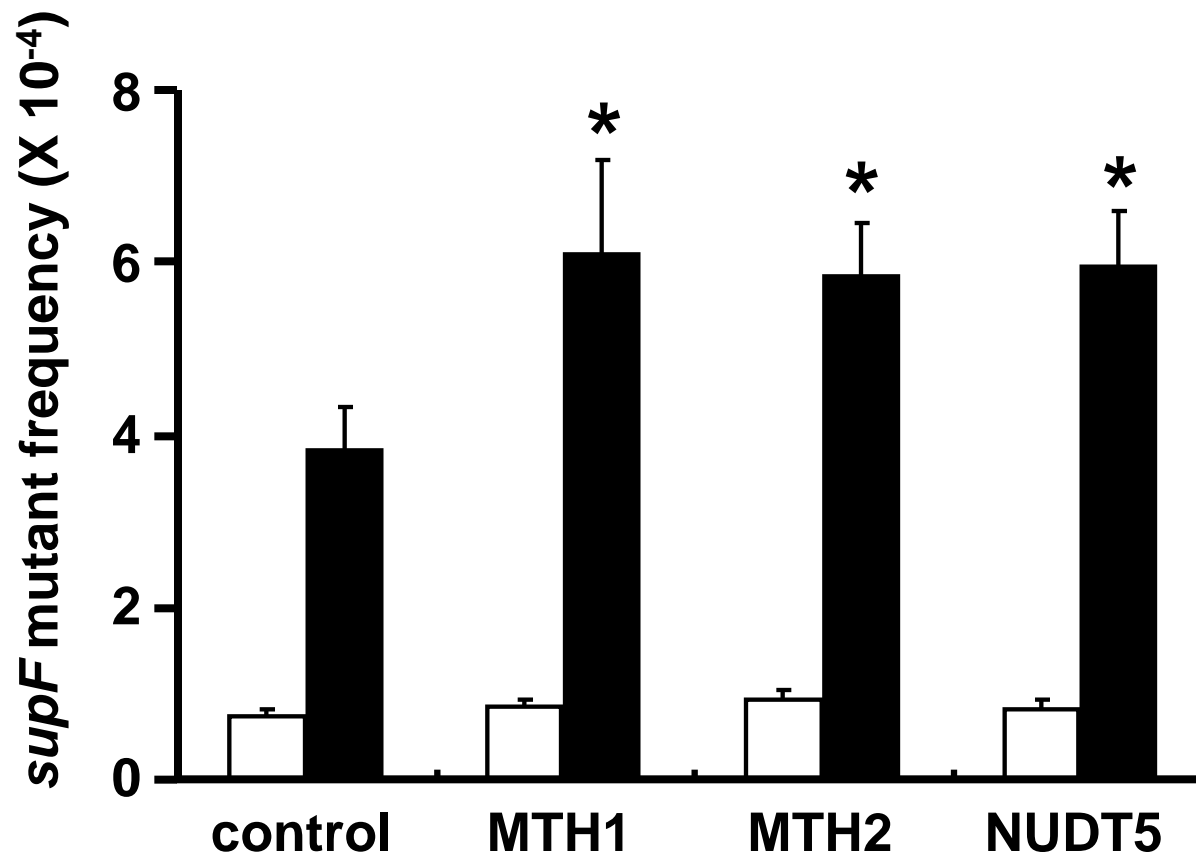
- [35] Kamiya, H.; Yakushiji, H.; Dugué, L.; Tanimoto, M.; Pochet, S.; Nakabeppu, Y.; Harashima, H. Probing the substrate recognition mechanism of the human MTH1 protein by nucleotide analogs. *J. Mol. Biol.* **336**:843-850; 2004.
- [36] Satou, K.; Kasai, H.; Masutani, C.; Hanaoka, F.; Harashima, H.; Kamiya, H. 2-Hydroxy-2'-deoxyadenosine 5'-triphosphate enhances A•T→C•G mutations caused by 8-hydroxy-2'-deoxyguanosine 5'-triphosphate by suppressing its degradation upon replication in a HeLa extract. *Biochemistry* **46**:6639-6646; 2007.
- [37] Yoshimura, D.; Sakumi, K.; Ohno, M.; Sakai, Y.; Furuichi, M.; Iwai, S.; Nakabeppu, Y. An oxidized purine nucleoside triphosphatase, MTH1, suppresses cell death caused by oxidative stress. *J. Biol. Chem.* **278**:37965-37973; 2003.
- [38] Rai, P.; Onder, T.T.; Young, J.J.; McFaline, J.L.; Pang, B.; Dedon, P.C.; Weinberg, R.A. Continuous elimination of oxidized nucleotides is necessary to prevent rapid onset of cellular senescence. *Proc. Natl. Acad. Sci. U.S.A.* **106**:169-174; 2009.
- [39] Furuta, A.; Iida, T.; Nakabeppu, Y.; Iwaki, T. Expression of hMTH1 in the hippocampi of control and Alzheimer's disease. *Neuroreport* **12**:2895-2899; 2001.
- [40] Zheng, J.-D.; Hei, A.-L.; Zuo, P.-P.; Dong, Y.-L.; Song, X.-N.; Takagi, Y.; Sekiguchi, M.; Cai, J.-P. Age-related alterations in the

- expression of MTH2 in the hippocampus of the SAMP8 mouse with learning and memory deterioration. *J. Neurol. Sci.* In press; 2009.
- [41] Yu, Y.; Cai, J.-P., Tu, B.; Wu, L.; Zhao, Y.; Liu, X.; Li, L., McNutt, M.A.; Feng, J.; He, Q.; Yang, Y.; Wang, H.; Sekiguchi, M.; Zhu, W.-G. Proliferating cell nuclear antigen is protected from degradation by forming a complex with MutT Homolog2. *J. Biol. Chem.* **284**:19310-19320; 2009.
- [42] Hori, M.; Asanuma, T.; Inanami, O.; Kuwabara, M.; Harashima, H.; Kamiya, H. Effects of overexpression and antisense RNA expression of Orf17, a MutT-type enzyme. *Biol. Pharm. Bull.* **29**:1087-1091; 2006.
- [43] Egashira, A.; Yamauchi, K.; Yoshiyama, K.; Kawate, H.; Katsuki, M.; Sekiguchi, M.; Sugimachi, K.; Maki, H.; Tsuzuki, T. Mutational specificity of mice defective in the MTH1 and/or the MSH2 genes. *DNA Repair (Amsterdam)* **11**:881-893; 2002.

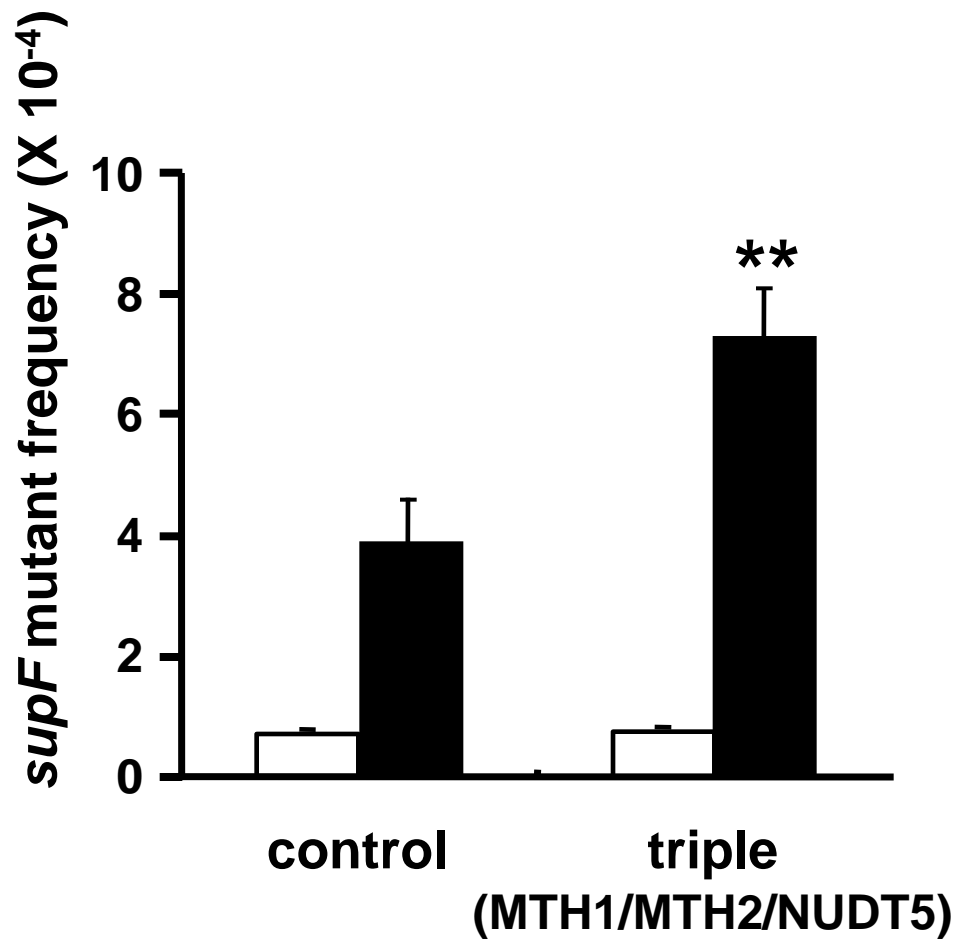
## Figure legends

**Fig. 1.** Effects of knock-down of MTH1, MTH2, and NUDT5 on 8-OH-dGTP-induced mutant frequency. Open columns, dGTP introduction; closed columns, 8-OH-dGTP introduction. Data are expressed as means  $\pm$  SEM (n=6 except for control siRNA, dGTP (n=5)). \* $P$ <0.05 vs control.

**Fig. 2.** Effects of triple knock-down of the MutT-type proteins on 8-OH-dGTP-induced mutant frequency. Open columns, dGTP introduction; closed columns, 8-OH-dGTP introduction. Data are expressed as means  $\pm$  SEM (n=5). \*\* $P$ <0.01 vs control.



**Fig. 1**



**Fig. 2**

**Table 1 Amounts of mRNAs in cells with knocked-down nucleotide pool sanitization enzymes**

<b>siRNA</b>	<b>48 h</b>	<b>72 h</b>
<b>MTH1</b>	<b>0.21 ± 0.02 (79%)</b>	<b>0.27 ± 0.03 (73%)</b>
<b>MTH2</b>	<b>0.24 ± 0.03 (76%)</b>	<b>0.32 ± 0.04 (68%)</b>
<b>NUDT5</b>	<b>0.10 ± 0.02 (90%)</b>	<b>0.17 ± 0.03 (83%)</b>

The amount of mRNA was measured by quantitative RT-PCR at 48 h and 72 h after siRNA introduction. The amount of mRNA was normalized relative to that of the human GAPDH mRNA contained in each sample. Relative values to that in 293T cells treated with the control siRNA are shown. Data are expressed as means  $\pm$  SEM (n=4). The values in parentheses show knock-down efficiencies.

**Table 2 Mutations observed upon dGTP treatment in cells with knocked-down nucleotide pool sanitization enzymes**

<b>Mutations</b>	<b>Control</b>	<b>MTH1</b>	<b>MTH2</b>	<b>NUDT5</b>
<b>Single base substitution</b>				
<b>Transition</b>				
<b>A:T → G:C</b>	<b>1 (6)</b>	<b>2 (13)</b>	<b>0 (0)</b>	<b>1 (5)</b>
<b>G:C → A:T</b>	<b>3 (17)</b>	<b>1 (6)</b>	<b>6 (24)</b>	<b>1 (5)</b>
<b>Transversion</b>				
<b>A:T → T:A</b>	<b>1 (6)</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>1 (5)</b>
<b>A:T → C:G</b>	<b>1 (6)</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>0 (0)</b>
<b>G:C → T:A</b>	<b>1 (6)</b>	<b>2 (13)</b>	<b>8 (32)</b>	<b>2 (10)</b>
<b>G:C → C:G</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>1 (4)</b>	<b>0 (0)</b>
<b>Deletions/Insertions</b>				
<b>1-2 bases</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>2 (8)</b>	<b>0 (0)</b>
<b>≥ 3 bases</b>	<b>8 (44)</b>	<b>6 (38)</b>	<b>5 (20)</b>	<b>14 (67)</b>
<b>Others</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>1 (4)<sup>a</sup></b>	<b>0 (0)</b>
<b>No mutation</b>	<b>3 (17)</b>	<b>5 (31)</b>	<b>2 (8)</b>	<b>2 (10)</b>
<b>Total</b>	<b>18 (100)</b>	<b>16 (100)</b>	<b>25 (100)</b>	<b>21 (100)</b>

All data are represented as cases found (%).

<sup>a</sup> A CG → ATA mutation at positions 117 and 118.

**Table 3 Mutations induced by 8-OH-dGTP in cells with knocked-down nucleotide pool sanitization enzymes**

<b>Mutations</b>	<b>Control</b>	<b>MTH1</b>	<b>MTH2</b>	<b>NUDT5</b>
<b>Single base substitution</b>				
<b>Transition</b>				
<b>A:T → G:C</b>	<b>0 (0)</b>	<b>1 (2)</b>	<b>1 (2)</b>	<b>0 (0)</b>
<b>G:C → A:T</b>	<b>4 (11)</b>	<b>0 (0)</b>	<b>8 (15)</b>	<b>5 (8)</b>
<b>Transversion</b>				
<b>A:T → T:A</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>1 (2)</b>	<b>0 (0)</b>
<b>A:T → C:G</b>	<b>25 (66)</b>	<b>47 (90)</b>	<b>36 (68)</b>	<b>50 (76)</b>
<b>G:C → T:A</b>	<b>1 (3)</b>	<b>1 (2)</b>	<b>3 (6)</b>	<b>3 (5)</b>
<b>G:C → C:G</b>	<b>2 (5)</b>	<b>2 (4)</b>	<b>1 (2)</b>	<b>0 (0)</b>
<b>Deletions/Insertions</b>				
<b>1-2 bases</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>0 (0)</b>
<b>≥ 3 bases</b>	<b>4 (11)</b>	<b>1 (2)</b>	<b>2 (4)</b>	<b>5 (8)</b>
<b>Others</b>	<b>2 (5)<sup>a</sup></b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>2 (3)<sup>b</sup></b>
<b>No mutation</b>	<b>0 (0)</b>	<b>0 (0)</b>	<b>1 (2)</b>	<b>1 (2)</b>
<b>Total</b>	<b>38 (100)</b>	<b>52 (100)</b>	<b>53 (100)</b>	<b>66 (100)</b>

All data are represented as cases found (%).

<sup>a</sup> TC → CA (at positions 110 and 111) and complex mutations.

<sup>b</sup> CTCT → C (at positions 93-96) and complex mutations.



**Table 4** Amounts of mRNAs in 293T cells with knocked-down nucleotide pool sanitization enzymes (triple knock-down)

siRNA	48 h	72 h
<b>Triple (MTH1 + MTH2 + NUDT5)</b>		
<b>MTH1</b>	<b>0.28 ± 0.06 (72%)</b>	<b>0.41 ± 0.03 (59%)</b>
<b>MTH2</b>	<b>0.31 ± 0.12 (69%)</b>	<b>0.56 ± 0.12 (44%)</b>
<b>NUDT5</b>	<b>0.16 ± 0.03 (84%)</b>	<b>0.25 ± 0.07 (75%)</b>

The amount of mRNA was measured by quantitative RT-PCR at 48 h and 72 h after siRNA introduction. The amount of mRNA was normalized relative to that of the human GAPDH mRNA contained in each sample. Relative values to that in 293T cells treated with the control siRNA are shown. Data are expressed as means ± SEM (n=3). The values in parentheses show knock-down efficiencies.

**Table 5 Mutations induced by 8-OH-dGTP in cells with knocked-down nucleotide pool sanitization enzymes (triple knock-down)**

Mutations	Control		Triple	
	dGTP	8-OH-dGTP	dGTP	8-OH-dGTP
<b>Single base substitution</b>				
<b>Transition</b>				
A:T → G:C	1 (3)	1 (1)	5 (11)	3 (3)
G:C → A:T	7 (18)	2 (2)	8 (18)	1 (1)
<b>Transversion</b>				
A:T → T:A	2 (5)	0 (0)	1 (2)	1 (1)
A:T → C:G	1 (3)	85 (81)	0 (0)	93 (86)
G:C → T:A	6 (16)	1 (1)	5 (11)	2 (2)
G:C → C:G	6 (16)	2 (2)	10 (22)	0 (0)
<b>Deletions/insertions</b>				
1-2 bases	0 (0)	1 (0)	0 (0)	1 (1)
≥ 3 bases	11 (29)	11 (10)	10 (22)	4 (4)
<b>Others</b>	0 (0)	0 (0)	2 (4) <sup>a</sup>	0 (0)
<b>No mutation</b>	4 (11)	2 (2)	4 (9)	3 (3)
<b>Total</b>	<b>38 (100)</b>	<b>105 (100)</b>	<b>45 (100)</b>	<b>108 (100)</b>

All data are represented as cases found (%).

<sup>a</sup> CC → TT (at positions 71 and 72) and GG → TC (at positions 84 and 85) mutations.