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NDX-1 protein hydrolyzes 8-oxo-7, 8-dihydrodeoxyguanosine-5′-diphosphate to sanitize oxidized nucleotides and prevent oxidative stress in *Caenorhabditis elegans*

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8-oxo-dGTP is generated in the nucleotide pool by direct oxidation of dGTP or phosphorylation of 8-oxo-dGDP. It can be incorporated into DNA during replication, which would result in mutagenic consequences. The frequency of spontaneous mutations remains low in cells owing to the action of enzymes degrading such mutagenic substrates. *Escherichia coli* MutT and human MTH1 hydrolyze 8-oxo-dGTP to 8-oxo-dGMP. Human NUDT5 as well as human MTH1 hydrolyze 8-oxo-dGDP to 8-oxo-dGMP. These enzymes prevent mutations caused by misincorporation of 8-oxo-dGTP into DNA. In this study, we identified a novel MutT homolog (NDX-1) of *Caenorhabditis elegans* that hydrolyzes 8-oxo-dGDP to 8-oxo-dGMP. NDX-1 did not hydrolyze 8-oxo-dGTP, 2-hydroxy-dATP or 2-hydroxy-dADP. Expression of NDX-1 significantly reduced spontaneous A:T to C:G transversions and mitigated the sensitivity to a superoxide-generating agent, methyl viologen, in an *E. coli mutT* mutant. In *C. elegans*, RNAi of *ndx-1* did not affect the lifespan of the worm. However, the sensitivity to methyl viologen and menadione bisulfite of the *ndx-1*-RNAi worms was enhanced compared with that of the control worms. These facts indicate that NDX-1 is involved in sanitization of 8-oxo-dGDP and plays a critical role in defense against oxidative stress in *C. elegans*.

Keywords: *Caenorhabditis elegans*/NDX-1/8-oxo-dGDPase/mutations/oxidative stress/sanitization.

Abbreviations: AP, apurinic/apyrimidinic; BER, base excison repair; *C. elegans*, *Caenorhabditis elagans*; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; HPLC, high-performance liquid chromatography; MV, methyl viologen; NDX, Nudix; RNAi, RNA interference; ROS, reactive oxygen species; 8-oxoG, 8-oxo-7, 8-dihydroguanine; 8-oxo-dGTP, 8-oxo-7, 8-dihydrodeoxyguanosine-5′-triphosphate; 8-oxo-dGDP, 8-oxo-7, 8-dihydrodeoxyguanosine-5′-diphosphate; 8-oxo-dGMP, 8-oxo-7, 8-dihydrodeoxyguanosine-5′-monophosphate; 2-oh-dATP, 2-hydroxy-5′-deoxyadenosine triphosphate; 2-oh-dADP, 2-hydroxy-5′-deoxyadenosine diphosphate.

Reactive oxygen species (ROS) are continually generated during normal cellular metabolism and are also produced by exposure to ionizing radiation and various chemical oxidizing agents. ROS generate oxidatively damaged purine and pyrimidine bases in DNA (*1*, *2*). Among these damaged bases, 8-oxo-7, 8-dihydroguanine (8-oxoG) is the most abundant and important in mutagenesis and carcinogenesis (*3*–*5*). 8-oxoG can be generated not only by direct oxidation of guanine in DNA but also by incorporation of 8-oxo-dGTP, an oxidized form of dGTP generated by ROS in the nucleotide pool, during DNA replication (*6*–*8*). It can be incorporated opposite adenine or cytosine of template DNA, causing A:T to C:G or G:C to T:A transversions (*1*, *7*, *9*–*11*).

Bacteria and eukaryotes are equipped with two types of elaborate mechanisms to prevent mutations by 8-oxoG. One is base excision repair (BER) for oxidatively damaged bases in DNA. In *Escherichia coli*, MutM removes 8-oxoG paired with cytosine in DNA, while MutY removes adenine incorporated opposite 8-oxoG in the template during DNA replication (*5*, *6*, *12*). These DNA glycosylases hydrolyse the *N*-glycosylic bond between the modified base and deoxyribose, thus releasing a free base and generating an apurinic/apyrimidinic (AP) site in DNA (*13*, *14*). The resulting AP sites are further processed during BER (*5*, *14*, *15*). Similar BER processes have been identified in bacteria, yeast and mammalian cells (*10*, *16*–*19*). Another error-avoiding process for preventing replication errors has also been revealed. The error-avoiding mechanism functions at the pre-replication step by degrading naturally occurring mutagenic substrates, such as 8-oxo-dGTP and 8-oxo-dGDP (*7*, *9*). MutT is involved in the prevention of mutations by 8-oxoG in *E. coli*. MutT and its human homolog MTH1 (hMTH1) have 8-oxo-dGTP pyrophosphatase (8-oxo-dGTPase) activity that hydrolyzes 8-oxo-dGTP

to 8-oxo-dGMP and PPi (*9*, *20*, *21*). Furthermore, MutT and human NUDT5 have 8-oxo-dGDP pyrophosphatase (8-oxo-dGDPase) activity that hydrolyzes 8-oxo-dGDP to 8-oxo-dGMP (*21–23*). 8-oxo-dGMP is not rephosphorylated to the triphosphate form and therefore cannot be incorporated into DNA by DNA polymerases (*7*). However, 8-oxo-dGDP is phosphorylated by nucleoside diphosphate kinase to generate 8-oxo-dGTP, which can be incorporated into DNA (*7*, *24*). Therefore, the 8-oxo-dGDPase activity of *E. coli* MutT and human NUDT5 also plays an important role in sanitization of the nucleotide pool to prevent mutations.

Caenorhabditis elegans is a multicellular eukaryote that is frequently used as a model animal for the study of mechanisms of development and aging (*25*). *Caenorhabditis elegans* is also used to study the relationship between oxidative stress and development and aging (*26*, *27*). Therefore, it is necessary to elucidate the mechanisms of repair of oxidative DNA damage and sanitization of oxidized nucleotides in *C. elegans*. We have identified *C. elegans* homologs of uracil DNA glycosylase and endonuclease III (NTH) (*28*, *29*). Recently, Arczewska *et al.* (*30*) demonstrated that NDX-4 of *C. elegans* exhibits the two hallmarks of a MutT-type enzyme, namely, the ability to hydrolyze 8-oxo-dGTP and to suppress *E. coli mutT* mutator phenotypes. In addition, a *C. elegans ndx-4* mutant showed increased sensitivity to a superoxide-generating drug, methyl viologen (MV). Interestingly, they also found that the *ndx-4* crude extract retained an additional 8-oxo-dGTPase activity (*30*). The nature and functions of this enzyme were not determined. Furthermore, proteins with 8-oxo-dGDPase activity have not been identified in *C. elegans*.

In this study, we identified a homologue of *E. coli* MutT in *C. elegans* (NDX-1). The *C. elegans* NDX-1 potently reduced the spontaneous A:T to C:G transversions and mitigated the sensitivity to MV in an *E. coli mutT* mutant. Purified NDX-1 protein had 8-oxo-dGDPase activity that was efficiently stimulated by Mg^{2+}. NDX-1 did not have pyrophosphatase activity for 8-oxo-dGTP, or for oxidized dATP or dADP. These results suggest that NDX-1, like human NUDT5, is involved in the sanitization of 8-oxo-dGDP generated in the nucleotide pool in *C. elegan*. There was no significant difference in lifespan between *ndx-1*-RNAi and control worms. However, the sensitivity to MV and menadione bisulfite of the *ndx-1*-RNAi worms was higher than that of the control worms. These results suggest that NDX-1 is involved in sanitization of 8-oxo-dGDP in the nucleotide pool and plays a critical role in defense against oxidative stress in *C. elegans*.

Materials and Methods

Cloning, expression and purification of NDX-1 of C. elegans

The database was searched for proteins with homology to *E. coli* MutT and human MTH1 using a BLAST search. The T26E3.2 (*ndx-1*), W02G9.1 (*ndx-2*), Y38A8.1 (*ndx-3*), Y37H9A.6 (*ndx-4*) and F13H10.2 (*ndx-9*) clones were detected. The *C. elegans ndx-1*, ndx-2, ndx-3,ndx-4 and ndx-9 cDNA clones were amplified by PCR from a cDNA library using the following primers: for *ndx-1*, 5′-CCA AGCGAATTCATGCCACTTGGAAAGTTG-3′ (forward with an *Eco*RI site) and 5′-AGGCTCGAGTTAAAGCATATGAAGTGA CGG-3′ (reverse with an *Xho*I site); for *ndx-2*, 5′-GGAATTCCGA ATTCACGTCATCGGCCACATCGCCAACCA-3′ (forward with an *Eco*RI site) and 5′-ATGCGGCCGCCTAGATCGTGGCGAA TTGGATGCCCA-3′ (reverse with a *Not*I site); for *ndx-3*, 5′- CCA AGCGAATTCTCTGTTCTGATTCCG-3′ (forward with an *Eco*RI site) and 5′-AGGGAAAAAAGCGGCCGCTTAGAAAAATTTC ACAATTAAAT-3′ (reverse with a *Not*I site), for *ndx-4*, 5′-ATAA GCGAATTCGTCGTAAAAGCCGCG-3′ (forward with an *Eco*RI site) and 5′-AATCTCGAGTTAAAACCGGCCAAAAAAGCGGA A-3′ (reverse with a *Xho*I site) and for *ndx-9*, 5′-CCAAGCGAATTC CCGACATTTCGAAGCA-3′ (forward with an *Eco*RI site) and 5′-AGGGAAAAAAGCGGCCGCCTAAATTCTAGAATTATG GTTT-3′ (reverse with a *Not*I site). pGEX4T-1 plasmid (Pharmacia Biotech) was used as a cloning and expression vector. The PCR products were cloned into pGEX4T-1 digested with appropriate restriction enzymes, and *E. coli* strain CC101 *mutT* was transformed with the resultant plasmid (pGEX*ndx-1*, pGEX*ndx-2*, pGEX*ndx-3*, pGEX*ndx-4* or pGEX*ndx-9*). The sequence of the inserts was checked to verify that no mutations had been introduced by the PCR.

Assay for spontaneous mutations of Lac$^+$ in E. coli

E. coli CC101 *mutT* cells carrying pGEX4T-1, pGEX*ndx-1*, pGEX*ndx-2*, pGEX*ndx-3*, pGEX*ndx-4* or pGEX*ndx-9* were grown at 37°C to stationary phase in 5 ml of LB medium containing 100 g/ml ampicillin. Appropriate dilutions of each culture were spread onto LB plates and minimal plates (10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g trisodium citrate, 1 mM $MgSO_4$ and 0.001% thiamine per liter) containing lactose (0.2%), followed by incubation at 37°C for 42 h. Spontaneous mutation frequencies were calculated by dividing the number of Lac^+ revertants by that of total viable cells.

Assay for the sensitivity of E. coli to MV and H_2O_2

Escherichia coli CC101/pGEX4T-1, CC101 *mutT*/pGEX4T-1 or CC101 *mutT*/pGEX*ndx-1* was grown in 5 ml of LB containing 100 μg/ml ampicillin at 37°C to stationary phase. Appropriate dilutions of each culture were plated on LB plates containing MV or H_2O_2 at various concentrations. After incubation at 37°C for 20 h, the number of colonies was counted to estimate survival.

Assay for enzymatic activity

A single colony of *E. coli* CC101 *mutT* carrying pGEX*ndx-1* was incubated in 20 ml of LB containing 100 μg/ml ampicillin and grown overnight at 37°C. Fifteen millilitres of the overnight culture were added to 1.5 litres of fresh LB (1:100 dilution) containing 100 μg/ml ampicillin and grown at 37°C until the optical density at 600 nm reached about 0.4. The culture was further incubated for 24 h at 15°C with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The induced cells were harvested, washed and resuspended in 60 ml of phosphate- buffered saline (PBS, pH 7.4) containing 0.1% Triton X-100. The cell suspension was sonicated and the cell lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was applied to a glutathione (GSH)–Sepharose 4B column (Pharmacia Biotech) that had been equilibrated with PBS. Thrombin was then added at 5 units/ml to the column to cleave the GST. After 48 h, purified NDX-1 was eluted from the column with PBS, followed by dialysis overnight at 4°C against PBS. The fractions containing the purified protein were pooled and stored at −80°C in 25% (v/v) glycerol until use. Proteins were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

8-oxo-dGTP and 8-oxo-dGDP were purchased from TriLink Biotechnologies (San Diego, CA) and Cosmo Bio (Tokyo, Japan), respectively. 2-oh-dATP was obtained from Chemgenes Corp. (Wilmington, NA). 2-oh-dADP was prepared by the oxidation of dADP. Reaction mixtures (25 μl) containing 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 40 mM NaCl, 8 mM DTT, 5% glycerol and each substrate at 10 μM were incubated at 37°C for 15 min with purified NDX-1. The reaction was terminated by adding 25 μl of 5 mM EDTA. The mixture was subjected to HPLC using a TSK-GEL DEAE-2SW column (Tosoh, Tokyo) at a flow rate of 1.0 ml/min for the mobile phase buffer (75 mM sodium phosphate, pH 6.0, 1 mM EDTA and 20% acetonitrile). The substrates and reaction products were detected by measuring UV absorbance at 254 nm for 2-oh-dATP, 2-oh-dADP, dGMP, dGDP and dGTP, or

293 nm for 8-oxo-dGMP, 8-oxo-dGDP and 8-oxo-dGTP. To examine the metal ion requirement, the reaction mixture contained $MnCl_2$, $ZnCl_2$, $CaCl_2$ or $MgCl_2$ at 4 mM.

Caenorhabditis elegans strains and culture conditions

Caenorhabditis elegans N2, a standard wild-type strain, was obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). *C. elegans* worms were cultured at 20°C on 90 mm NGM agar plates (0.3% NaCl, 0.25% polypeptone, 0.005% cholesterol, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM potassium phosphate, pH 6.0 and 0.17% agar) with a lawn of *E. coli* OP50.

Bacteria-mediated RNAi of ndx-1

The *C. elegans ndx-1* cDNA was amplified by PCR. The primers used for PCR were 5′-ATCTTCTAGAGATGCCACTTGGAAAG T-3′ (forward with an *Xba*I site) and 5′-CTATCTGCAGTTAAAGC ATATGAAGTGA-3′ (reverse with a *Pst*I site).The amplified PCR products were subcloned into plasmid L4440 for bacteria-mediated feeding-RNAi (RNAi plasmid). *E. coli* HT115 (DE3) was transformed with the RNAi plasmid. For RNAi experiments, the transformants (instead of *E. coli* OP50) were plated on NGM plates containing 1 mM IPTG (RNAi plates). L4440, an empty vector, was used for a negative control of RNAi.

RNA preparation and RT-PCR analysis

ndx-1-RNAi and control worms were collected and RNA was extracted from them using TriPure Isolation Reagent (Roche). Total RNA was isolated using a High Pure RNA Tissue Kit (Roche) according to the manufacturer's instructions. cDNA was synthesized using ReverTra Ace (Toyobo) and used for RT-PCR. The quantity of cDNA was corrected using *tbg-1* as an internal standard. After 30–40 cycles of amplification, the RT-PCR products were separated by electrophoresis on 1% agarose gels.

Assay for lifespan of C. elegans

Worms were harvested by rinsing with S buffer (100 mM NaCl, 50 mM potassium phosphate, pH 6.0) and resuspended in alkaline hypochlorite (500 mM NaOH and 1.2% hypochlorite) until the adults dissolved (5–10 min). Eggs were then washed five times with S buffer. The eggs were transferred to NGM plates or RNAi-plates, allowed to develop to adults (about 3 days) and moved to fresh NGM plates or RNAi plates (8–10 worms/plate) supplemented with 40 M 5-fluoro-2′-deoxyuridine to suppress the production of progeny (*31*). The day when young adults were moved was taken as day 0, and the living populations were counted every 2 days. RNAi worms were moved to new RNAi plates every 6 days. Whether a nematode was alive or dead was judged by its response to tapping it on the plate. The experiments were performed at 20°C.

Assay for the sensitivity of C. elegans to oxidative stress

Eggs were placed on RNAi plates containing MV, menadione bisulfite or H_2O_2 at various concentrations. After incubation at 20°C for 4 days, L4 and adult worms were counted (smaller worms were not counted) and the number of L4 and adults/the number of eggs was determined.

Results

Identification and cloning of C. elegans ndx-1, ndx-2, ndx-3, ndx-4 and ndx-9 genes

To identify MutT homolog(s) in *C. elegans*, we searched for proteins with homology to *E. coli* MutT and hMTH1. Five candidate proteins, NDX-1, NDX-2, NDX-3, NDX-4 and NDX-9, were detected using a BLAST search. These proteins contained a sequence homologous to the Nudix motif (Fig. 1), which is conserved among MutT-related proteins from *E. coli* MutT to hMTH1 (*21*, *32*). *C. elegans* NDX-1, NDX-2, NDX-3, NDX-4 and NDX-9 contained 365, 223, 188, 138 and 348 amino acids, respectively. The degrees of sequence identity/similarity between *C. elegans* NDX and MutT were 38%/75% for NDX-1, 43%/70% for NDX-2, 29%/69% for NDX-3, 31%/65% for NDX-4 and 22%/58% for NDX-9.

Complementation of spontaneous mutations and high sensitivity to MV in an E. coli mutT *by NDX-1*

The cDNA clones encoding NDX-1, NDX-2, NDX-3, NDX-4 and NDX-9 were amplified by PCR from a *C. elegans* cDNA library. Each PCR fragment was inserted into a pGEX4T-1 expression vector, and *E. coli* CC101 *mutT* was transformed with each of the plasmids (pGEX*ndx-1*, pGEX*ndx-2*, pGEX*ndx-3*, pGEX*ndx-4* or pGEX*ndx-9*). Disruption of the *mutT* gene leads to about a 1,000-fold increase in spontaneous mutation frequency due to A:T to C:G transversions in *E. coli* (*9*). Mutation frequency was determined by using $LacZ^+$ reversion to clarify whether NDX1, NDX-2, NDX-3, NDX-4 and NDX-9 were able to complement the spontaneous mutations in an *E. coli mutT* mutant. The frequency of mutation leading to reversion to $LacZ^+$ in *E. coli* CC101 *mutT* was potently reduced by expressing NDX-1, compared with that of the *mutT* mutant carrying an empty vector (Fig. 2A). Expression of NDX-4 also significantly reduced the spontaneous mutation frequency in *E. coli* CC101 *mutT*, while expression of NDX-2 had only a partial effect on the mutation frequency (Fig. 2A). Expression of NDX-3 or NDX-9 was not able to reduce the mutation frequency (data not shown), as previously described (*30*). The mutant *lacZ* gene on the F′ episome in *E. coli* CC101 *mutT* reverts to the wild-type Lac^+ only as a result of A:T to C:G transversion and not as a result of any other substitutions (*33*). From these results, it is clear that NDX-1 could suppress A:T to C:G transversions, like *E. coli* MutT and hMTH1.

Previous studies showed that *mutT*-deficient bacteria are more sensitive to ROS than the wild-type strains. In *Bacillus subtilis*, loss of *mutT* sensitizes growing cells to H_2O_2 (*34*). *Pseudomonas aeruginosa mutT* strain is significantly more sensitive than the wild-type to H_2O_2 (*35*). In this study, the sensitivities of *E. coli* strains to MV and H_2O_2 were assessed by plating the overnight cultures on LB plates containing MV or H_2O_2, followed by determination of the survival. *E. coli* CC101 *mutT* mutant was more sensitive to MV than the wild-type strain (Fig. 2B). To clarify whether NDX-1 complements the sensitivity of an *E. coli mutT* mutant to MV, we compared the sensitivity of *E. coli* CC101 *mutT* to MV with that of wild-type CC101 and CC101 *mutT* bearing the vector alone (Fig. 2B). *E. coli* CC101 *mutT* expressing NDX-1 was more resistant to MV compared with CC101 *mutT*. Thus, it was clear that NDX-1 mitigated the sensitivity of *E. coli mutT* to MV. *E. coli* CC101 *mutT* mutant was not more sensitive to H_2O_2 than the wild-type strain (Fig. 2C).

These results indicated that NDX-1 of *C. elegans* has MutT-type abilities to suppress spontaneous mutations and to play a role in preventing oxidative stress. Arczewska *et al.* (*30*) had already demonstrated that NDX-4 of *C. elegans* efficiently hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate. Therefore, in this study, we focused on the properties

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MutT      1      --------------------------MKKLQIAVGIIRNENNEIFITRRAADAHMANKLE
MTH1      1      --------------------------MGASRLYTLVLVLQPQRVLLGMKKRGFGAGRWN
NDX-1     46     VAKKKNEPRVPDMQLGKCRYVRLHDNVNYVAAAIILRNQGDDTEVLLIQEAKKSCRGKWY
NDX-2     48     VGFKTHTGQVGVWQSVHRNTKPVEASADGVSIIARVRKQGKLYIVLVKQYRIPCGKLCLE
NDX-3     1      --------------------------MSVLIPLVTVDGRDSVLLTKRSIHLRSHRGEVC
NDX-4     1      --------------------------MVVKAAGLVIYRKLAGKIEFLLLQASYPPHHWT
NDX-9     155    CPKCAAALKMRSSKSGAECVTCQRVYYPTFSPVSITLITDPTNEHALLVRHRGSAGGVFT

MutT      35     FPGGKIEMGETPEQAVVRELQEEVGITPQHFSLFEKLEYEFPDRHITLWFWLVERWEGEP
MTH1      34     GFGGKVQEGETIEDGARRELQEESGLTVDALHKVGQIVFEFVGEPELMDVHVFCTDSIQG
NDX-1     106    MPAGRVEAGETIEEAVVREVKEETGYSCDVVELLSLQVQGSGWYRYAFYCNITGGDLKTE
NDX-2     108    LPAGLIDAGETAQQAAIRELKEETGYVSGKVVMESKLCFLDPGLTDDSQCLVVVDVDGDA
NDX-3     34     FPGGRMDPGETTTETALRETFEEIGVNAESVEIWGHLKSVIRRQADFNVTPIVGYISDER
NDX-4     34     PPKGHVDPGEDEWQAAIRETKEEANITKEQLTIHEDCHETLFYEAKGKPKSVKYWLAKLN
NDX-9     215    AVAGFAHSGESMAECARREIAEEVGIEVDSIRSLDMSQPWPMPDSSLMIAHVAVAKIDQK

MutT      95     WGKEGQPGEWMSLVGLNADDFPPANEPVIAKLKRL--------------------------
MTH1      94     TPVESDEMRPCWFQLDQIPFKDMWPDDSYWFPLLLQKKKFHGYFKFQGQDTILDYTLREV
NDX-1     166    PDQESLAAEWYNIKDLKANKVQLRGRDFIRLVDEAVTYRTHGPVDSIPRVMPLNQNVAGL
NDX-2     168    PENQNPVQVLDSTESIEVLLVEQSALMAYVTNLDSSSIVVESTLLAYAMGIQFATI----
NDX-3     94     VLENLVVNSDEVQAVFTIPIDELIKKAGLTKFQSKRMKYTLPSFDSTEFKVHHNAPNEYL
NDX-4     94     NPDDVQLSHEHQNWKWCELEDAIKIADYAEMGSLLRKFSAFLAGF---------------
NDX-9     275    ISVCPDELETAQWFTRHQVKEALTTTLADPLLKNLPRTLDDRQTLHYIPPAGAIAHQMIR
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Fig. 1 Amino acid sequence alignment of *C. elegans* NDX-1, *C. elegans* NDX-2, *C. elegans* NDX-3, *C. elegans* NDX-4, *C. elegans* NDX-9, *E. coli* MutT and human MTH1. Nudix motifs and Nudix motif-like sequences are indicated by the box.

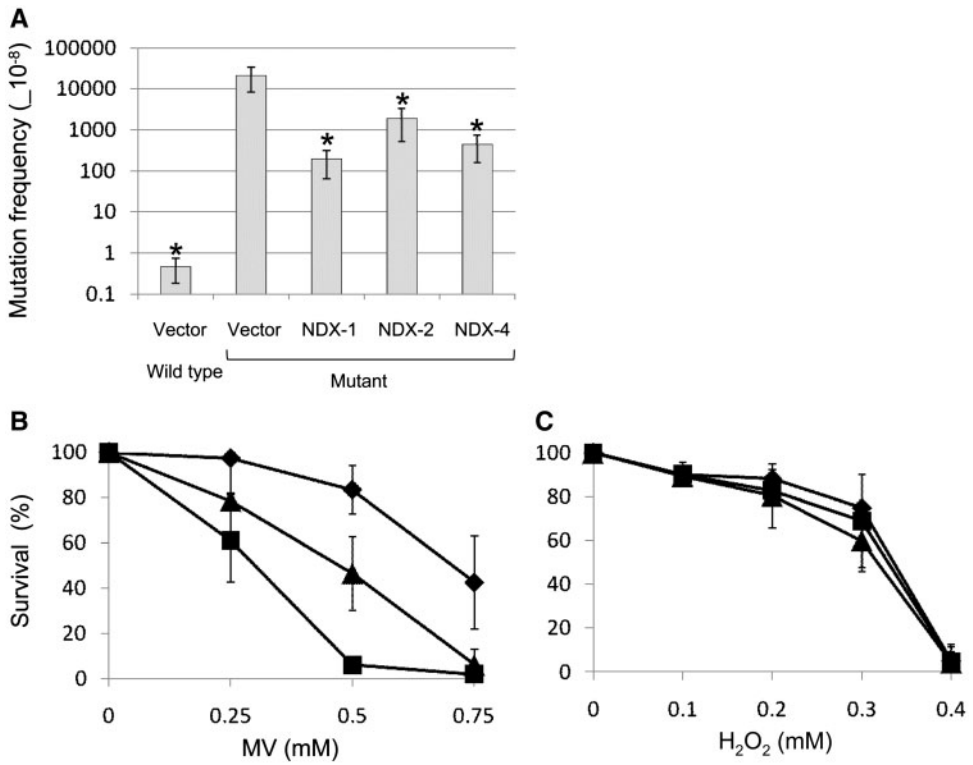

Fig. 2 Complementation assay. (A) Complementation of the mutator phenotype in *E. coli mutT* mutant (CC101 *mutT*) by expressing NDX-1, NDX-2 or NDX-4 protein. Mutation frequencies were determined by LacZ$^+$ reversion. (B and C) The sensitivity of *E. coli* CC101, CC101 *mutT* and CC101 *mutT* expressing NDX-1 to MV and H_2O_2. The overnight cultures were plated on LB agar plates containing MV (B) and H_2O_2 (C) and the number of colonies was counted to estimate survival after 20 h of incubation at 37°C. The values represent the mean ± standard deviation ($n = 4$). *$P < 0.05$ vs *E.coli mutT* mutant, indicating significant difference at the 95% confidence level. Filled diamond, *E. coli* CC101; (filled square) *E. coli* CC101 *mutT*; (filled triangle) *E. coli* CC101 *mutT* with pGEX*ndx-1*.

of NDX-1 of *C. elegans* as an *E. coli* MutT homolog with 8-oxo-dGTPase and/or 8-oxo-dGDPase activity.

Purification and enzymatic activity of C. elegans NDX-1

Caenorhabditis elegans NDX-1 was expressed as a GST-fused protein in *E. coli* CC101 *mutT* to avoid contamination with *E. coli* MutT protein during purification. The GST-NDX-1 fusion protein was purified by glutathione-Sepharose column chromatography, and the GST-tag was removed with thrombin. NDX-1 had an apparent molecular weight of 45 kDa (Fig. 3A).

We assayed the enzymatic activity of purified NDX-1 towards various substrates as described in *Materials and Methods* section. The reaction mixtures were subsequently subjected to high-performance liquid chromatography (HPLC). The HPLC profiles are shown in Fig. 3B. Without NDX-1, peaks corresponding to 8-oxo-dGDP and 8-oxo-dGTP appeared at 18 min and 23 min of retention time, respectively (Figs. 3B(1) and 3B(3)). After incubation with NDX-1 at 37°C for 15 min, the area of the peak at 18 min markedly decreased and the peak at 13 min corresponding to 8-oxo-dGMP increased (Fig. 3B(2)). On the other hand, the area of the peak at 23 min was not changed (Fig. 3B(4)). The results shown in Fig. 3C indicate that NDX-1 is able to hydrolyze 8-oxo-dGDP, but not 8-oxo-dGTP, to the monophosphate form. NDX-1 did not hydrolyze 2-oh-dATP or 2-oh-dADP (Table I). In addition, it did not hydrolyze normal dGTP, dGDP, dATP or dADP (Table I).

Table I. Hydrolysis of oxidized and normal nucleotides by *C. elegans* NDX-1.

Substrate	Hydrolysis (%)
dGTP	<2
dGDP	<2
8-oxo-dGTP	<2
8-oxo-dGDP	61.5
dATP	<2
dADP	<2
2-oh-dATP	<2
2-oh-dADP	<2

Each substrate at 10 μM was incubated with purified NDX-1 (0.2 μg) at 37°C for 30 min in the presence of 4 mM $MgCl_2$.

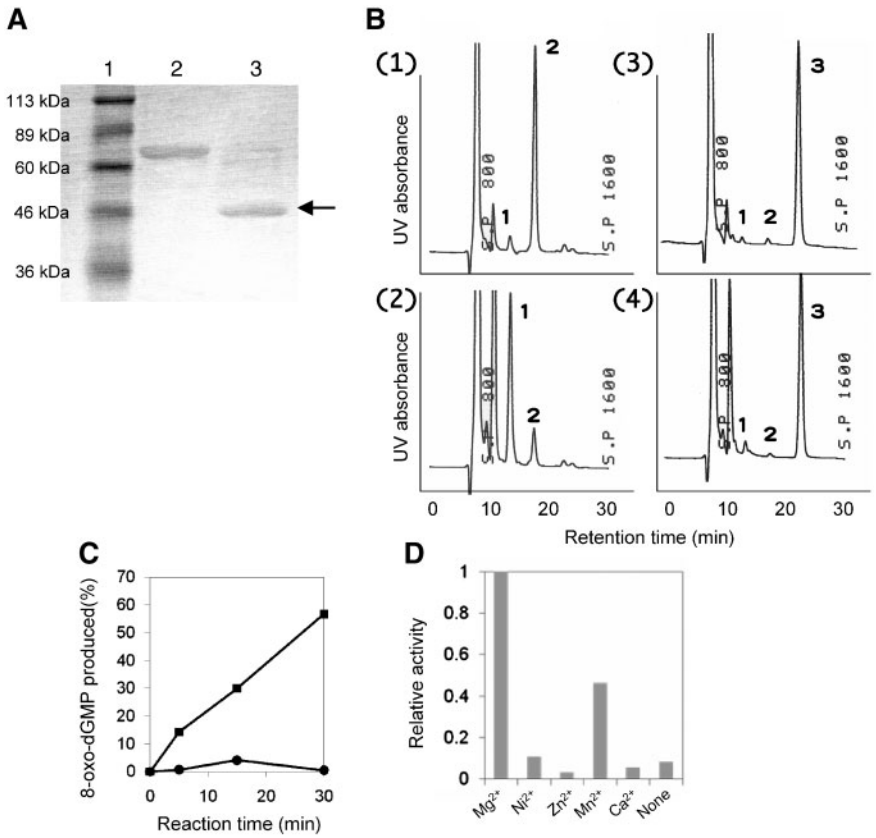

Fig. 3 Cleavage activity of NDX-1 against 8-oxo-dGDP and 8-oxo-dGTP. (**A**) Purification of NDX-1 protein. Proteins were analysed by SDS–PAGE (12% polyacrylamide) and stained with Coomassie Brilliant blue R 250. Lane 1, molecular weight markers; lane 2, purified NDX-1 GST-fusion protein; lane 3, purified NDX-1 protein after cleavage with thrombin. The arrows indicate the purifed NDX-1. (**B**) Substrate specificity of NDX-1. 8-oxo-dGDP (B(1) and B(2)) or 8-oxo-dGTP (B(3) and B(4)) after incubation at 37°C for 15 min without (B(1) and B(3)) or with (B(2) and B(4)) 0.2 μg of purified NDX-1. The elution times of 8-oxo-dGMP, 8-oxo-dGDP and 8-oxo-dGTP are indicated by **1**, **2** and **3**, respectively. (**C**) Time course. 8-oxo-dGDP or 8-oxo-dGTP was incubated with 0.1 μg of purified NDX-1 for the indicated incubation time at 37°C. (Filled square) 8-oxo-dGDP; (filled circle) 8-oxo-dGTP. (**D**) The metal ion requirement of NDX-1. Reaction mixtures containing various metal ions (4 mM) were incubated at 37°C for 15 min with 0.1 μg of purified GST-NDX-1 fusion protein. Relative activity was plotted on the vertical axis.

Next, the metal ion requirement of the enzyme was examined. $MgCl_2$, $MnCl_2$, $ZnCl_2$ or $CaCl_2$ was added to the reaction mixture at 4 mM. Among these metal ions, Mg^{2+} stimulated the activity of NDX-1 most effectively. Mn^{2+} gave 47% of the activity obtained in the presence of Mg^{2+}, whereas there was no detectable hydrolysis activity with Zn^{2+} or Ca^{2+} (Fig. 3D).

Physiological effects of the knockdown of ndx-1 by RNAi in C. elegans

To examine the physiological roles of NDX-1, we examined whether the knockdown of *ndx-1* affects the lifespan and the sensitivity to ROS using the well-established RNAi method (*36*, *37*). To confirm the effect of *ndx-1*-RNAi, RT-PCR was carried out with total RNA prepared from *ndx-1*-RNAi and control worms. *ndx-1* mRNA was depleted in *ndx-1*-targeted worms as compared with the control worms (Fig. 4A). *Ndx-2*, *ndx-3*, *ndx-4* and *ndx-9* mRNAs were not depleted, while the expression of *ndx-8* was slightly reduced (see Supplementary Fig. S1). The lifespan of *ndx-1*-RNAi worms did not differ from that of control worms (Fig. 4B).

Next, we compared the sensitivity of *ndx-1*-RNAi worms to MV, menadione bisulfite and H_2O_2 by determining the percent of an embryos that developed to adults after 4 days on plates containing each of these agents in *ndx-1*-RNAi and control worms. The *ndx-1*-RNAi worms showed higher sensitivity to MV and menadione bisulfite compared with the control worms (Figs. 4C and 4D). On the other hand, the *ndx-1*-RNAi worms did not show higher sensitivity to H_2O_2 (Fig. 4E). These results were in accord with the finding that NDX-1 efficiently complemented the sensitivity of *E. coli mutT* mutant to MV, but not to H_2O_2 (Figs. 2B and 2C).

Discussion

The expression of NDX-1 significantly reduced the frequency of spontaneous A:T to C:G transversions in an *E. coli mutT* mutant (Fig. 2A), as does the expression of *E. coli* MutT or human MTH1 (*1*, *24*, *38*). MutT and hMTH1 prevent mutations owing to their ability to hydrolyze mutagenic substrate 8-oxo-dGTP generated by ROS in the nucleotide pool (*7-9*, *20*). Hence, it was expected that NDX-1 would have 8-oxo-dGTPase activity, like *C. elegans* NDX-4 (*30*). However, purified NDX-1 was unable to hydrolyze 8-oxo-dGTP (Figs. 3C and 3D). 8-oxo-dGDP is generated by oxidation as efficiently as 8-oxo-dGTP in the nucleotide pool (*7*, *23*). The present experiments revealed that NDX-1 has the 8-oxo-dGDPase activity, as does human NUDT5 (*22*, *23*). Furthermore, both NDX-1 of *C. elegans* and human NUDT5 are capable of reducing the spontaneous mutation frequency in *E. coli mutT* to the wild-type level (Figs. 2A; *22*, *23*). Thus, it is possible that phosphorylation of 8-oxo-dGDP generated by ROS effectively produces the mutagenic substrate 8-oxo-dGTP in the nucleotide pool. Thus, the conversion of 8-oxo-dGDP to 8-oxo-dGTP might also be a potent inducer of mutations in *C. elegans*.

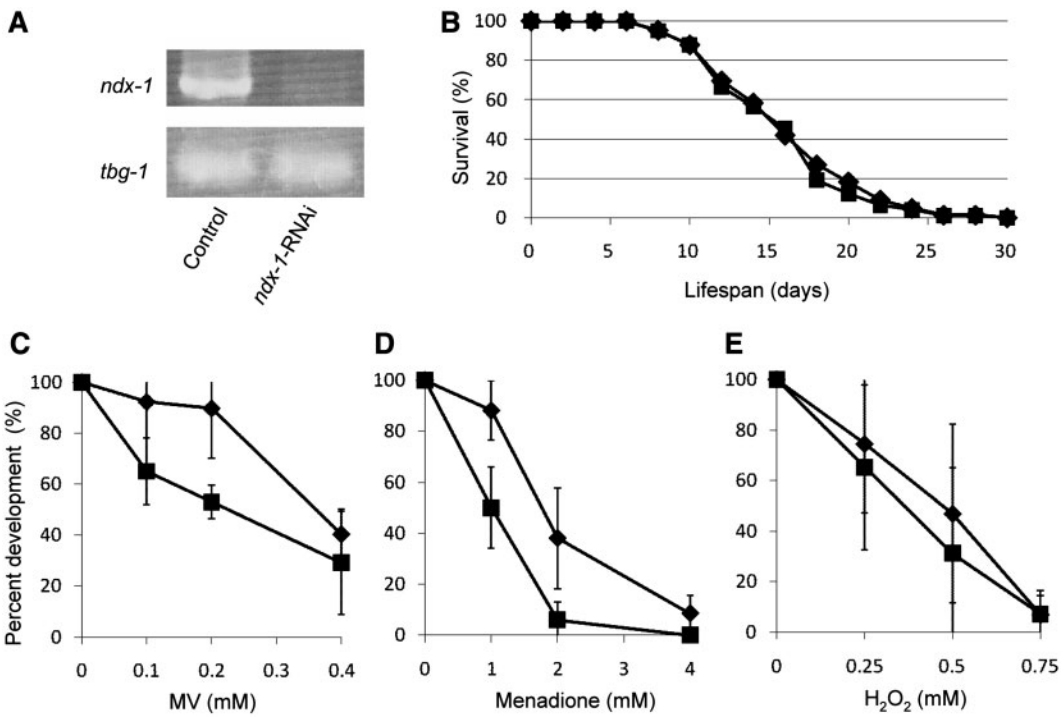

Fig. 4 The phenotypes of *C. elegans* subjected to RNAi of *ndx-1*. (A) RT-PCR of total RNA isolated from *ndx-1*-RNAi and control worms. *tbg*-1 was used as an internal standard. (B) Lifespan of *ndx-1*-RNAi and control worms. *ndx-1*-RNAi and control worms were cultured on agar plates at 20°C and RNAi worms were moved to new RNAi plates every 6 days. The surviving population was counted every 2 days. (Open square) *C. elegans* N2; (filled suare), *C. elegans ndx-1* RNAi. (C–E) The sensitivity of *ndx-1*-RNAi worms and control worms to MV (C), menadione bisulfite (D) and H_2O_2 (E). The values indicate the ratio of the number of viable L4 and adults/the number of eggs. The values represent the mean ± standard deviation ($n = 3$). (Open square), *C. elegans* N2; (filled square), *C. elegans ndx-1* RNAi.

Recently, Arczewska *et al*. (*30*) demonstrated that NDX-4 of *C. elegans* has abilities to both hydrolyze 8-oxo-dGTP and suppress *E. coli mutT* mutator phenotypes. NDX-4 degrades 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate. It does not degrade 8-oxo-dGDP. 8-oxo-dGDP is also produced by the action of NDX-4, but with less efficiency than 8-oxo-dGMP (*30*). In summary, the conversion of 8-oxoG-containing nucleotides in *C. elegans* is illustrated in Fig. 5. 8-oxo-dGTP is generated not only by oxidation of dGTP but also by phosphorylation of 8-oxo-dGDP by nucleotide diphosphate kinase (*7*). 8-oxo-dGTP is effectively degraded by the 8-oxo-dGTPase activity of NDX-4 (*30*), as it is by hMTH1 (*7*, *8*, *24*). Furthermore, 8-oxo-dGDP is degraded by NDX-1, as it is by human NUDT5 (*22*, *23*). 8-oxo-dGTP can be incorporated into nascent DNA by DNA polymerases and would thereby induce mutations. 8-oxo-dGMP is not rephosphorylated; instead, it is further degraded by 8-oxo-dGMPase, yet unidentified enzyme, in *C. elegans*. DNA glycosylases that excise 8-oxoG residues from DNA have not yet been identified in *C. elegans*. Efficient elimination of 8-oxo-dGTP and 8-oxo-dGDP from the nucleotide pool may contribute to overcoming the potent mutagenic activity of 8-oxoG.

It is possible that *C. elegans* possesses additional proteins with pyrophosphatase activity towards 8-oxo-dGTPase and/or 8-oxo-dGDPase. In fact, Arczewska *et al*. (*30*) showed that residual activity that can degrade 8-oxo-dGTP is present in the extract prepared from the *ndx-4* null mutant *C. elegans*. Moreover, other protein(s) in addition to NDX-1 may possess 8-oxo-dGDPase activity in *C. elegans*. It is very important to identify such proteins in *C. elegans*. We are currently attempting to identify such proteins by purification using column chromatography. In addition to MutT, *E. coli* has another type of 8-oxo-dGTPase, GTP cyclohydrase II, encoded by the ribA gene (*39*). This protein is not a member of the MutT family, but can hydrolyze 8-oxo-dGTP. Thus, there is a different pathway from the MutT pathway for degrading 8-oxo-dGTP and 8-oxo-dGDP. An attempt to identify such proteins with 8-oxo-dGTPase activity is also underway in order to fully clarify the mechanisms of sanitization of oxidized nucleotides in *C. elegans*.

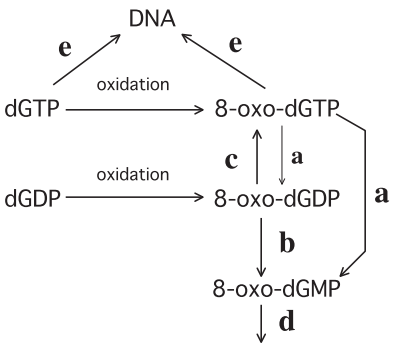

Fig. 5 Conversion of 8-oxoG-containing nucleotides dGTP in *C. elegans*. This scheme is based on the study of Sekiguchi and Tsuzuki (*7*). (**a**) 8-oxo-dGTPase (NDX-4); (**b**) 8-oxo-dGDPase (NDX-1); (**c**) nucleoside diphosphate kinase; (**d**); 8-oxo-dGMPase (unidentified); (e) DNA polymerase; (**e**) DNA polymerase.

Most MutT-related proteins, such as *E. coli* MutT and human MTH1, have a common amino acid motif (GGKx12REx2EE), which is essential for 8-oxo-dGTPase activity (*32*, *40*, *41*). Among these seven amino acid residues, two residues (G37 and K39) of *E. coli* MutT (*32*) are different from those of NDX-1 (A108 and R110). This difference might account for of the fact that NDX-1 does not have 8-oxo-dGTPase activity. Further studies will be required to elucidate the relationship between the substrate specificity and the amino acid sequence of MutT-related 8-oxo-dGTPases.

The biological significance of MutT-related proteins has been well documented. MTH1-null mouse embryo fibroblasts are highly susceptible to cell dysfunction and death caused by exposure to H_2O_2 (*42*). Furthermore, tumors are formed at higher incidence in the lung, liver and stomach of MTH1-null mice compared with the wild-type mice (*43*). The *E. coli mutT* mutant (CC101 *mutT*) was more sensitive to MV than the wild-type strain (Fig. 2B). This might have been due to a lack of the ability to eliminate oxidized nucleotides generated by MV. These findings strongly suggest that oxidized nucleotides, if not eliminated, cause cell death, mutagenesis and carcinogenesis. Recent studies using *C. elegans* have provided some of the best correlative evidence that oxidative stress caused by ROS plays a role in lifespan determination (*26*, *27*). It is neccesary to clarify the mechanisms underlying the relationship between oxidative stress and aging. We are currently studying whether there is a correlation between the repair of oxidatively damaged bases in DNA and the sanitization of oxidized nucleotides in the nucleotide pool, and attempting to clarify the influence of the loss of these pathways on the lifespan of *C. elegans* (*28*, *29*). In this study, RNAi of the *ndx-1* gene did not result in severe phenotypes of growth or development in *C. elegans*. RNAi of *ndx-1* did not affect lifespan compared with that of control worms (Fig. 4B). Assessment of the lifespan and mutagenesis in *ndx-1 ndx-4* double mutants of *C. elegans* is now underway in our laboratory.

Furthermore, *ndx-1*-RNAi worms were more sensitive to MV and menadione bisulfite compared with control worms (Figs. 4C and 4D), but were not more sensitive to H_2O_2 (Fig. 4E). These results were in accord with the fact that the expression of NDX-1 could mitigate the sensitivity of an *E. coli mutT* mutant to MV but not to H_2O_2 (Figs. 2B and 2C). This difference might be explained by the high reactivity of superoxide generated by MV and menadione with the nucleotides. In summary, it is likely that NDX-1 plays a role in defense against oxidative stress.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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