An increase of oxidized nucleotides activates DNA damage checkpoint pathway that regulates post-embryonic development in *Caenorhabditis elegans*

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ABSTRACT

8-oxo-dGTP, an oxidized form of dGTP generated in the nucleotide pool, can be incorporated opposite adenine or cytosine in template DNA, which can in turn induce mutations. In this study, we identified a novel MutT homolog (NDX-2) of *Caenorhabditis elegans* (*C. elegans*) that hydrolyzes 8-oxo-dGDP to 8-oxo-dGMP. In addition, we found that NDX-1, NDX-2 and NDX-4 proteins have 8-oxo-GTPase or 8-oxo-GDPase activity. The sensitivity of *ndx-2* knockdown *C. elegans* worms to methyl viologen and menadione bisulfite was increased compared with that of control worms. This sensitivity was rescued by depletion of *chk-2* and *clk-2*, indicating that growth of the worms is regulated by the checkpoint pathway in response to the accumulation of oxidized nucleotides. Moreover, we found that the sensitivity to menadione bisulfite of *ndx-1* and *ndx-2* knockdown worms was enhanced by elimination of XPA-1, a factor involved in nucleotide excision repair (NER), suggesting that incorporated oxidized nucleotides are processed by a pathway in which XPA-1 functions.

INTRODUCTION

Oxidative damage to bases occurs through normal cellular metabolism and through exposure to ionizing radiation and various chemical oxidizing agents (1). Among such damaged bases, an oxidized form of guanine, 8-oxo-7,8-dihydroguanine (8-oxoG), can alter genetic information since it pairs with adenine and cytosine (2, 3). 8-oxo-dGTP, an oxidized form of dGTP generated in the nucleotide pool, is incorporated opposite adenine or cytosine of template DNA, causing A:T to C:G or G:C to T:A transversions (4, 5).

To prevent such deleterious effects, *Escherichia coli* (*E. coli*) MutT degrades 8-oxo-dGTP and 8-oxo-dGDP to 8-oxo-dGMP, and thereby prevents incorporation of the 8-oxoG-containing nucleotide (1, 6). Mammalian cells also possess enzymes that eliminate 8-oxoG-containing nucleotides from the nucleotide pool. These include MTH1 (5, 7), MTH2 (NUDT15) (8), MTH3 (NUDT18) (9) and NUDT5 (10). MTH1 and MTH2 preferentially degrade 8-oxodGTP, whereas NUDT5 and MTH3 hydrolyse 8-oxo-dGDP but show little activity toward 8-oxo-dGTP. In *Caenorhabditis elegans* (*C. elegans*), two MutT homologs, NDX-1 and NDX 4, have been identified (11, 12). NDX-1 cleaves 8-oxo-dGDP but not 8-oxo-dGTP, while NDX-4 cleaves 8-oxo-dGTP but not 8-oxo-dGDP. In this study, we searched for another MutT homolog of *C. elegans* and found a novel hydrolase for 8-oxo-dGDP encoded by *ndx-2*.

Furthermore, it has been shown that 8-oxoG can be incorporated into RNA by RNA polymerase (13). 8-oxo-GTP is incorporated opposite adenine in the DNA template during transcription and the altered transcripts could lead to mistranslated proteins. *E.coli* MutT protein has the ability to degrade both 8-oxo-GDP and 8-oxo-GTP in the nucleotide pool (14, 15). Many MutT homologs have hydrolytic activity toward 8-oxo-GTP or 8-oxo-GDP (9, 16). Thus, we investigated whether the NDX proteins act on 8-oxoG-containing ribonucleotides.

Eukaryotes respond to DNA damage by activating the DNA damage response (DDR) pathway (17). The DDR includes DNA repair processes and checkpoint pathways that regulate cell cycle progression and affect development, growth rate, life span and sensitivity to DNA damage (18). In *C. elegans*, checkpoint pathways are conserved and DNA damage checkpoint genes such as *chk-2* and *clk-2* have been described (19). The CLK-2 checkpoint protein has been found to be involved in the activation of cell-cycle arrest and apoptosis in response to misincorporation of uracil (20). Interaction between the base-excision repair (BER) pathway and activation of the DDR signaling pathway through CLK-2 has been described (20). We examined whether the DDR pathway is

activated in response to an increased amount of oxidized nucleotides or their misincorporation. Here, we report that CHK-2 and CLK-2 checkpoint proteins function in postembryonic development and growth control.

If incorporated into DNA, the 8-oxoG lesion should be repaired. Bacteria and eukaryotes are equipped with mechanisms to prevent mutations by 8-oxoG (21). One such mechanism is base excision repair (BER) for oxidatively damaged bases in DNA. In *E. coli*, MutM removes 8-oxoG paired with cytosine in DNA from DNA (21, 22). In yeast and mammalian cells, OGG1 has been shown to remove 8-oxoG to initiate BER (23, 24). In contrast, little is known about repair pathway(s) processing 8-oxo-G in DNA in *C. elegans*. *C. elegans* lacks homologs of *E. coli* MutM and mammalian OGG1. We carried out a survey of repair pathways that would act on 8-oxoG-containing nucleotides. Here, we report that XPA-1, which is a factor acting in nucleotide excision repair (NER), seems to be involved in repair of incorporated 8-oxoG, and that activation of the checkpoint pathway is partially linked to the function of XPA-1.

MATERIALS AND METHODS

Assay for the sensitivity of *E.coli* to oxidative stress

Plasmids used in this experiment were previously documented (11). *E. coli* CC101 harboring pGEX4T-1, CC101 *mutT* harboring pGEX4T-1 or CC101 *mutT* expressing NDX-2 was grown to stationary phase in 5 ml of LB containing 100 μg/ml ampicillin at 37°C. Appropriate dilutions of each culture were plated on LB plates containing methyl viologen (MV). After incubation at 37°C for 20 hr, the number of colonies was counted to estimate survival.

Assay for enzymatic activity

C. elegans NDX-1, NDX-2 and NDX-4 were purified as previously described (11). 8-oxo-dGTP was purchased from TriLink Biotechnologies (San Diego, CA) and 8-oxo-dGDP and 8-oxo-GTP were purchased from Cosmo Bio (Tokyo, Japan). 8-oxo-GDP was prepared as the product of hydrolysis of 8-oxo-GTP by NDX-4. Reaction mixtures (25 μl) containing 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 40 mM NaCl, 8 mM dithithreitol, 5% glycerol and each substrate at 20 μM were incubated at 37°C for 30 min with purified NDX

protein. The reaction was terminated by adding 25 μl of 5 mM EDTA. The mixture was subjected to HPLC (high performance liquid chromatography) 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 dGMP, dGDP, dGTP, GMP, GDP and GTP, or 293 nm for 8-oxo-dGMP, 8-oxo-dGDP, 8-oxo-dGTP, 8-oxo-GMP, 8-oxo-GDP and 8-oxo-GTP.

***C. elegans* strains and culture conditions**

C. elegans N2, RB1054 *ndx-4* (*ok1003*), RB1572 *mlh-1* (*ok1917*), RB877 *nth-1* (*ok724*) and RB864 *xpa-1* (ok698) were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). *C. elegans* worms were cultured at 20°C on 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 RNA interference (RNAi)

For knockdown experiments, we used the well-established RNAi method (25, 26). *C. elegans ndx-1*, *ndx-2*, *chk-2* (Y60A3A.12) and *clk-2* (C07H6.6) cDNA clones were amplified by PCR from a cDNA library using the following primers: for *ndx-1*, 5'-CCAAGCAAGCTTCCACTTGGAAAGTTG-3' (forward with a Hind III site) and 5'- AGGCTCGAGTTAAAGCATATGAAGTGACGG -3' (reverse with an XhoI site); for *ndx-2*, 5'-AGGGAAAAAAGCGGCCGCACGTCATCGGCCACA-3' (forward with a Not I site) and 5'-CTATCTGCAGCTAGATCGTGGCGAATT-3' (reverse with a Pst I site); for *chk-2*, 5'-TTAAGCGCGGCCGCATGGTTCGCGGGACA-3' (forward with a NoI I site) and 5'-TTAAGCCTGCAGTCACATTTTTGCCTTTTTCACAG-3' (reverse with a PsII site); and for *clk-2*, 5'-TTAAGCAAGCTTATGAATTTACGAAGTCGCCT-3' (forward with a Hind III site) and 5'-TTAAGCCTCGAGTGCGTAATTGAGATCACTC-3' (reverse with an Xho I site); The amplified PCR products were subcloned into plasmid L4440 for bacteria-mediated feeding-RNAi (RNAi plasmid). Double RNAi experiments were performed by mixing equal amounts of overnight cultures of *E. coli* HT115 (DE3) that had been transformed with the respective RNAi plasmids and then plating the mixture on NGM plates containing

1 mM IPTG (RNAi plates). The transformant harboring L4440 was used for a negative control of RNAi and for single knockdown experiments. To confirm the effect of RNAi, RT-PCR was carried out with total RNA prepared from RNAi and control worms (Supplementary Figure 1).

Assay for survival of *C. elegans* in adulthood under oxidative conditions

To assay survival of *C. elegans* in adulthood, young adult worms were transferred to plates containing 5 mM menadione bisulfite and survival was monitored daily. This protocol was similar to the assay for lifespan (11), except that the plates contained 5 mM menadione bisulfite.

Assay for the sensitivity of *C. elegans* to oxidative stress

To assay sensitivity to menadione bisulfite during development, eggs were placed on RNAi plates containing MV or menadione bisulfite. After incubation at 20°C for 4 days, L4 and adult worms were counted and the number of L4 and adults/the number of eggs was determined. Smaller larvae were also counted on the previous day and the number of L1 to L3 larvae/the number of eggs was determined. Then, to determine growth in the first filial generation (F1) progeny animals, eggs were collected from the *ndx-4* mutants that developed to adulthood on plates containing 0 mM or 0.5 mM menadione bisulfite and placed on NGM plates with a lawn of *E. coli* OP50. After incubation for 4 additional days, L4 and adult worms were counted and the number of (L4 and adults)/the number of eggs was determined.

RESULTS

Complementation of sensitivity to methyl viologen (MV) of *E. coli mutT* by NDX-2

Previous studies revealed that there are at least two MutT homologs, NDX-1 and NDX 4, in *C. elegans* (11, 12). We first examined whether *C. elegans* possess additional proteins with pyrophosphatase activity towards 8-oxo-dGTP and/or 8-oxo-dGDP. *C. elegans* NDX-2 is predicted to be an ADP-sugar diphosphatase orthologous to human NUDT5 . NUDT5 also has 8-oxo-dGDPase activity and plays an important role in preventing mutations. Expression of NDX-2 was able to reduce the mutation frequency in *E. coli mutT*, as

previously described (11, 12). To examine whether NDX-2 complements the sensitivity of *E. coli mutT* mutants to methyl viologen (MV), we compared the survival of *E. coli* CC101 *mutT* expressing NDX-2 with that of wild-type CC101 and CC101 *mutT*. We found that *E. coli* CC101 *mutT* expressing NDX-2 was more resistant to MV compared with CC101 *mutT* bearing the vector alone (Supplementary Figure 2). Therefore, in this study, we first focused on the properties of NDX-2 of *C. elegans* as an *E. coli* MutT homolog.

Purification and enzymatic activity of *C. elegans* NDX-2

C. elegans NDX-2 was expressed as a GST-fused protein in *E. coli* CC101 *mutT*, the GST-NDX-2 fusion protein was purified by glutathione-Sepharose 4B column chromatography, and the GST-tag was removed with thrombin (Figure 1A). Assays for enzyme activities were carried out using 20 μM 8-oxo-dGDP or 8-oxo-dGTP as substrate, and the products were analyzed by HPLC. As shown in Figure 1B, NDX-2 degraded 8-oxo-dGDP to its monophosphate form, and hydrolysis of 8-oxo-dGTP was hardly detected. The substrate specificity of NDX-2 is shown in Table 1.

E.coli MutT protein and many MutT homologs also have activity toward the oxidized ribonucleotide, as reported previously (9, 14-16). We examined whether NDX-1, NDX-2 and NDX-4 also hydrolyze 8-oxo-GTP and/or 8-oxo-GDP. As shown in Figure 1C and Table 1, NDX-1 and NDX-2 showed 8-oxo-GDPase activity. Under the same conditions, hydrolysis of 8-oxoGTP by NDX-2 was hardly detected. NDX-4 showed 8-oxo-GTPase activity, while the hydrolysis of 8-oxoGDP was not detected.

The effect of superoxide generators on survival in *C elegans* treated in the adult stages

Previous studies showed that the lifespan of *C. elegans* is not affected by the knockdown of *ndx-1* or knockout of *ndx-4* (11, 12). The lifespan of *ndx-2*-RNAi worms was also similar to that of control worms (data not shown). We next examined whether oxidative stress affects the survival of *ndx*-1- and *ndx-2*-RNAi worms and *ndx-4* mutants. To test this, young adult worms were incubated on plates containing 5 mM menadione bisulfite, a superoxide generator. The survival was assessed by the percentage of live worms. While *ndx-1*-RNAi and *ndx-2*-RNAi worms showed similar survival to control worms, mutation of *ndx-4* decreased the survival of worms (Figure 2). Furthermore, double knockdown of *ndx-1* and *ndx-2* in *ndx-4* mutants showed significantly

lower survival (Figure 2). These results suggest that NDX-1, NDX-2 and NDX-4 co-ordinately participate in sanitization of the nucleotide pool.

Increased sensitivity to oxidative stress during postembryonic development in *C. elegans* by knockdown and knockout of the *ndx* genes

Previous studies showed that *ndx-4* mutants are slightly sensitive to PQ (MV) (12). *ndx-1*-RNAi worms also show higher sensitivity to MV and menadione bisulfite compared with control worms (11). We compared the sensitivity of *ndx-2*-RNAi and control worms to MV or menadione bisulfite by assessing the decrease in the growth rate of the worms. The eggs of the worms were exposed to the agents on plates for 4 days. The percent of eggs that developed to adults was determined as the growth rate. As shown in Figure 3A, like *ndx-1*-RNAi worms, *ndx-2*-RNAi worms showed higher sensitivity to both MV and menadione bisulfite than the control worms.

It is likely that NDX-1, NDX-2 and NDX-4 co-ordinately degrade 8-oxoG-containing nucleotides. To test this, we examined whether the knockdown or knockout of multiple *ndx* genes increased the sensitivity to MV and menadione bisulfite. In this study, we used the feeding RNAi method for the knockdown of *ndx-1* and *ndx-2*, and the *ndx-4* mutant for the knockout of *ndx-4*. As shown in Figure 3B, unlike the case of survival in adulthood, application of *ndx-1*- and/or *ndx-2*-RNAi did not Increase the sensitivity of the *ndx-4* mutant.

The effect of knockdown of *chk-2* and *clk-2* on the sensitivity to oxidative stress

Although the number of L4-adult stage worms was decreased in the plates containing menadione bisulfite, a few small larvae were observed in these plates (Figure 4A). This suggested that some of the worms that did not reach the adult stage underwent retarded development or arrest at the larval stage rather than death under the oxidative condition. We hypothesized that activation of checkpoint pathways caused this arrest. In *C. elegans*, various checkpoint pathways are conserved and DNA damage checkpoint genes such as *chk-2* and *clk-2* have been described (19). It is known that cell-cycle arrest and apoptosis are activated in response to dUTP misincorporation into DNA in *C. elegans* (20). To assess whether the growth-arrest involved CHK-2- and/or CLK2-mediated checkpoint pathways, we examined the effect of knockdown of *chk-2* and *clk-2* on the growth

rate of *ndx-1*- or *ndx-2*-RNAi worms or *ndx-4* mutants. While *ndx-1*- or *ndx-2*-RNAi worms or *ndx-4* mutants showed sensitivity to menadione bisulfite, as shown in Figure 3B, the number of worms that were able to develop to adulthood was restored to the control level by the knockdown of *chk-2* and *clk-2* (Figure 4A). That is, the sensitivity was rescued by the knockdown of *chk-2* and *clk-2*.

Despite the rescue of growth rate by the knockdown of *chk-2* and *clk-2*, DNA damage seemed not to be repaired, which could result in serious physiological problems. We therefore examined the influence on the reproduction of the *ndx-4* mutants that had evaded growth arrest at the larval stage and their progeny. All the adult worms in the *chk-2*- and *clk-2*-RNAi plates could lay eggs. We did not observe any obvious differences in the hatching rate compared to the control. We next examined the growth of the first filial generation (F1) progeny of the *chk-2*- and *clk-2*-RNAi worms. As shown in Figure 4B, the progeny of the *chk-2*- and *clk-2*-RNAi worms exhibited a lower growth rate than the progeny of the control-RNAi worms.

The effect of elimination of *xpa-1* on the sensitivity to oxidative stress

Once the abnormal nucleotides are incorporated, they should be removed and then repaired properly. Pathways including mismatch repair (MMR), base excision repair (BER) and nucleotide excision repair (NER) are conserved mechanisms for DNA repair in *C. elegans*. However, little is known about pathways for the repair of incorporated 8-oxoG in *C. elegans*. Multiple repair genes are conserved in *C. elegans*, including *mlh-1* (mismatch repair), *nth-1* (base excision repair) and *xpa-1* (nucleotide excision repair), and their knockout mutants, *mlh-1*(*ok1917*), *nth-1*(*ok724*) and *xpa-1*(*ok698*), are available. We examined whether the knockout of *mlh-1*, *nth-1* and *xpa-1* repair gene affects the sensitivity to menadione bisulfite in *ndx-1*- and *ndx-2*-RNAi worms. As shown in Figure 5A, the knockout of *mlh-1* and *nth-1* did not affect the sensitivity. On the other hand, the knockout of *xpa-1* increased the sensitivity of the *ndx-1*- and *ndx-2*-RNAi worms.

As described above, knockdown of *chk-2* and *clk-2* rescued the sensitivity of *ndx-1*- and *ndx-2*-RNAi worms. We next examined whether knockdown of *chk-2* and *clk-2* rescues the sensitivity of *ndx-1*- and *ndx-2*-RNAi *xpa-1* mutant. As shown in Figure 5B, the sensitivity was only slightly (not significantly) rescued by the knockdown of *chk-2* and *clk-2*.

DISCUSSION

MutT homologs that eliminate oxidized nucleotides have been identified in various organisms. In *C. elegans*, NDX-1 and NDX-4 selectively degrade 8-oxo-dGDP and 8-oxo-dGTP, respectively (11, 12). We first focused on the properties of NDX-2 of *C. elegans* as an *E. coli* MutT homolog. To examine the enzymatic activity of *C. elegans* NDX-2, the NDX-2 protein was purified (Figure 1A). Biochemical studies revealed that NDX-2, like NDX-1, hydrolyzes 8-oxo-dGDP, but not 8-oxo-dGTP, to the monophosphate form (Figure 1B). NDX-2 can hydrolyze normal dGDP as efficiently as 8-oxo-dGDP (Table 1). This property is similar to that of NDX-4 in that these enzymes hydrolyze normal nucleotides (12). The 8-oxo-dGDPase activity of NDX-2 was significantly lower than that of NDX-1. This fact might explain why the suppression of mutagenesis by the expression of NDX-2 was not as efficient as that by NDX-1 or NDX-4 (11, 12). However, NDX-1 and NDX-2 provide mutual backup in sanitization of the nucleotide pool. As reported previously, 8-oxodGDP strongly inhibits 8-oxo-dGTPase activity of MTH1 (27). Likewise, the presence of 8-oxo-dGDP or dGDP reduces NDX-4's 8-oxo-dGTPase activity (Supplementary Figure 3). The enzymatic activity of NDX-2 toward both 8-oxo-dGDP and dGDP might be necessary to avoid a reduction of the activity of NDX-4. 8-oxoG incorporation into RNA is considered to cause translational errors resulting in the synthesis of abnormal proteins (13). Although 8-oxoG in DNA is removed by DNA glycosylases such as MutM, OGG1 and NEIL1 to initiate BER and repair using the other strand (22-24, 28), 8-oxoG formed in RNA cannot be similarly removed. Therefore, organisms must be equipped with mechanisms to prevent transcriptional and translational errors caused by oxidative damage. MutT and many homologs of MutT also hydrolyze 8-oxoGTP and/or 8-oxo-GDP (9, 14, 16). In this study, we examined the enzymatic activity of NDX-1, NDX-2 and NDX-4 toward oxidized ribonucleotides and found that NDX-1 and NDX-2 had 8-oxo-GDPase activity, and NDX-4 had 8-oxo-GTPase activity. These facts suggest that NDX-1, NDX-2 and NDX-4 are also involved in sanitization of the ribonucleotide pool and play a critical role in preventing the synthesis of abnormal proteins. As shown in Figure 2, *ndx-1*, *ndx-2* double-RNAi *ndx-4* mutants displayed lower survival when placed on plates containing menadione bisulfite in adulthood. In *C. elegans*, the germ line is the only tissue in which cell divisions occur into adulthood (29). Therefore, the lower survival seems to have little to do with oxidatively damaged deoxyribonucleotides such as 8-oxo-dGTP. The lower survival may instead be explained by NDX's inability to sanitize the ribonucleotide pool.

We examined the effect of methyl viologen and menadione bisulfite on growth rate to assess the sensitivity during development. As shown in Figure 3A, knockdown of *ndx-2* caused sensitivity to both methyl viologen and menadione bisulfite. Although NDX-1, NDX-2 and NDX-4 may collaborate to prevent the

misincorporation of 8-oxoG, knockdown of *ndx-1* or *ndx-2* did not enhance the sensitivity of the *ndx-4* mutant (Figure 3B). We have, up to this point, determined the ratio of the number of viable L4 and adults, but a few small larvae were also observed in the plates containing the oxidizing agents. We hypothesized that some of the worms were arrested at the larval stage because the checkpoint pathways that regulate development are activated in response to an increase in the amount of oxidized nucleotides such as 8-oxo-dGTP (Figure 4A). To test this possibility, we examined the effect of the knockdown of *chk-2* and *clk-2*, DNA damage checkpoint genes in *C. elegans*, on the sensitivity of *ndx* knockdown or knockout worms. As shown in Figure 4A, the sensitivity was rescued by the knockdown of *chk-2* or *clk-2*. These results suggest that development is regulated by the function of CHK-2 and CLK-2. Checkpoint pathways are activated in response to dUTP misincorporation into DNA (20), and we speculate that the checkpoint pathways may also be activated in response to 8-oxo-dGTP misincorporation into DNA, although it is possible that the checkpoint pathways respond independently of misincorporation of 8-oxo-dGTP.

We next asked if adult worms that had evaded the growth arrest had some potential problem. To do this, we examined the effects on the reproduction of these adult worms and their progeny using the *ndx-4* mutant. Although there were no abnormalities in the hatching rate of eggs from the adult worms, the growth of the first filial generation (F1) progeny of the *chk-2*- and *clk-2*-RNAi worms was retarded compared to that of control-RNAi worms (Figure 4B). This result suggests that the checkpoint pathway is probably important to make sufficient time available for repair and to prevent transgenerational accumulation of DNA damage that would impair growth.

8-oxoG is generated in DNA by misincorporation of 8-oxo-dGTP. Therefore, it is also important to examine DNA repair pathways for 8-oxoG. The base excision repair (BER) pathway is the main mechanism for removal of 8-oxoG in DNA (30). Furthermore, mismatch repair (MMR) plays a role in the prevention of the mutagenic effect of 8-oxoG (31, 32). We examined whether the knockout of *nth-1* and *mlh-1* affects the sensitivity of *ndx-1*, *ndx-2* double-RNAi worms to menadione bisulfite and found there were no changes in the sensitivity. *C. elegans* lacks homologues of *E. coli* MutM and mammalian OGG1 that remove 8-oxoG from DNA. Although *C. elegans* NTH-1, a homolog of endonuclease III, was reported to have activity toward 8-oxoG paired with G, this activity is very weak (33). Therefore, we tested the possibility that another pathway is primarily involved in the repair of 8-oxoG in *C. elegans*. In yeast, nucleotide excision repair (NER) also contributes to the release of 8-oxoG in damaged DNA (32). It has been proposed that oxidative lesions relevant to aging are normally repaired by NER in *C. elegans* (34). Thus, we examined whether the knockout of *xpa-1*

affected the sensitivity of *ndx-1*, *ndx-2* double-RNAi worms to menadione bisulfite and found that knockout of *xpa-1* greatly increased the sensitivity (Figure 5A). XPA-1 is an orthologue of the human nucleotide excision repair gene XPA. It is generally reported that short-patch BER accounts for the majority of 8-oxoG repair and that NER may function in the repair of 8-oxoG. Reduced repair of oxidative DNA damage in XP-A cell extracts has previously been reported (35, 36). In *C. elegans*, the *xpa-1*(*ok698*) mutant is not hypersensitive to methyl viologen in adulthood and shows mild sensitivity to exposure to juglone, a superoxide generating agent (34, 37). The *xpa-1*(*ok698*) mutant was not sensitive to menadione bisulfite during development (Figure 5A). In contrast, the *ndx-1*- and *ndx-2*-double knockdown *xpa-1* mutant was hypersensitive to menadione bisulfite. Taking these results altogether, we concluded that XPA-1 is involved in repair of 8-oxoG, probably in a replication-coupled manner during development. Moreover, we assessed whether the increased sensitivity of the *xpa-1* mutant was rescued by knockdown of *chk-2* or *clk-2*. The sensitivity was slightly, but not significantly, rescued by the knockdown of *chk-2* or *clk-2*(Figure 5B). This result suggests that the *chk-2* and *clk-2* checkpoint pathway is at least partially linked to the function of XPA-1.

In this study, we investigated the physiological roles of NDX proteins and the response of *C. elegans* to an increased amount of oxidized nucleotides in the nucleotide pool. We identified factors that participate in the response to the oxidative DNA lesions. However, how they are functioning remains unclear, and the molecular mechanisms should be studied in detail.

SUPPLEMENTARY DATA

Supplementary Data are available at *Mutagenesis* Online.

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FIGURE LEGENDS

Figure 1. Cleavage activity of *C. elegans* NDX proteins against 8-oxoG-containing nucleotides. (A) Purification of NDX-2 and NDX-4. NDX-2 and NDX-4 were obtained by the cleavage of the GST-fused protein with thrombin. 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-2 or NDX-4 protein. The arrows indicate purified NDX-2 and NDX-4. (B) Substrate specificity of NDX-2. 8-oxo-dGDP or 8-oxo-dGTP after incubation at 37°C for 30 min without (top) or with (bottom) 5 μM purified NDX-2. 8-oxo-dGMP, 8-oxo-dGDP and 8-oxo-dGTP are indicated by (1), (2) and (3), respectively. (C) Substrate specificity of NDX proteins. 8-oxo-GDP or 8-oxo-GTP after incubation at 37°C for 30 min without or with 0.73 μM purified NDX-1, 5 μM NDX-2 or 1.51 μM NDX-4. 8-oxo-GMP, 8-oxo-GDP and 8-oxo-GTP are indicated by (1), (2) and (3), respectively.

Figure 2. The survival of *ndx-4*(*ok1003*) mutants in adulthood under oxidative conditions. *ndx-4*(*ok1003*) and *ndx-1*;*ndx-2* (double-RNAi)/*ndx-4* (*ok1003*) worms showed decreased survival on 5 mM menadione bisulfite plates compared to control worms, The surviving population was counted daily from day 3. ◆, control worms; ■, *ndx-4* (*ok1003*) worms; ▲, *ndx-1*;*ndx-2* (double-RNAi)/*ndx-4*(*ok1003*) worms. The values represent the mean ± standard deviation (n=3).

Figure 3. The sensitivity of *ndx-1*(RNAi) and *ndx-2*(RNAi) worms and *ndx-4*(*ok1003*) mutants to methyl viologen (MV) and menadione bisulfite. (A) *ndx-2*(RNAi) worms showed decreased development on MV and menadione bisulfite plates compared to control worms. ◆, control worms;▲, *ndx-2*(RNAi) worms. (B) *ndx-4*(*ok1003*) worms similarly exhibited sensitivity to MV and menadione bisulfite. Co-application of *ndx-1*(RNAi) and *ndx-2*(RNAi) did not enhance the sensitivity of the *ndx-4* mutant. ◆, control worms; ■, *ndx-4*(*ok1003*) worms; ▲, *ndx-1*(RNAi)/*ndx-4*(*ok1003*) worms; ●, *ndx-2*(RNAi)/*ndx-4*(*ok1003*) worms; ×, *ndx-1*;*ndx-2*(double-RNAi)/*ndx-4*(*ok1003*) worms. The ratio of the number of viable L4 and adults to the number of eggs was determined and normalized by the untreated control. The values represent the mean ± standard deviation (n=3).

Figure 4. Activation of checkpoint pathways in response to an increased amount of oxidized nucleotides. (A) Effect of knockdown of *chk-2* and *clk-2* on the sensitivity of *ndx-1*(RNAi) and *ndx-2*(RNAi) worms and *ndx-4* mutants to 1 mM bisulfite. The values indicate the number of viable L1 - L3/the number of eggs (white bar) and viable L4 and adults/the number of eggs (gray bar). The values represent the mean ± standard deviation (n=3). (B) Comparison of growth in the first filial generation (F1) progeny animals of the *ndx-4*(*ok1003*) mutants that were able to develop to adulthood under oxidative condition caused by menadione bisulfite (0.5 mM). The values represent the mean ± standard deviation (n=3). $*P < 0.05$, indicating significant difference at the 95% confidence level.

Figure 5. A survey of DNA repair pathways that process the incorporated oxidized nucleotides. (A) Effect of knockout of DNA repair genes on the sensitivity of *ndx-1*(RNAi) and *ndx-2*(RNAi) worms. (B) Effect of knockdown of *chk-2* and *clk-2* on the increased sensitivity of *ndx-1*(RNAi)/*xpa-1*(ok698) and *ndx-2*(RNAi)/*xpa-1*(ok698) knockdown worms to menadione bisulfite. The values are shown as the number of

viable L4 and adults/the number of eggs. The values represent the mean ± standard deviation (n=3). *$P < 0.05$, indicating significant difference at the 95% confidence level.

Figure 1

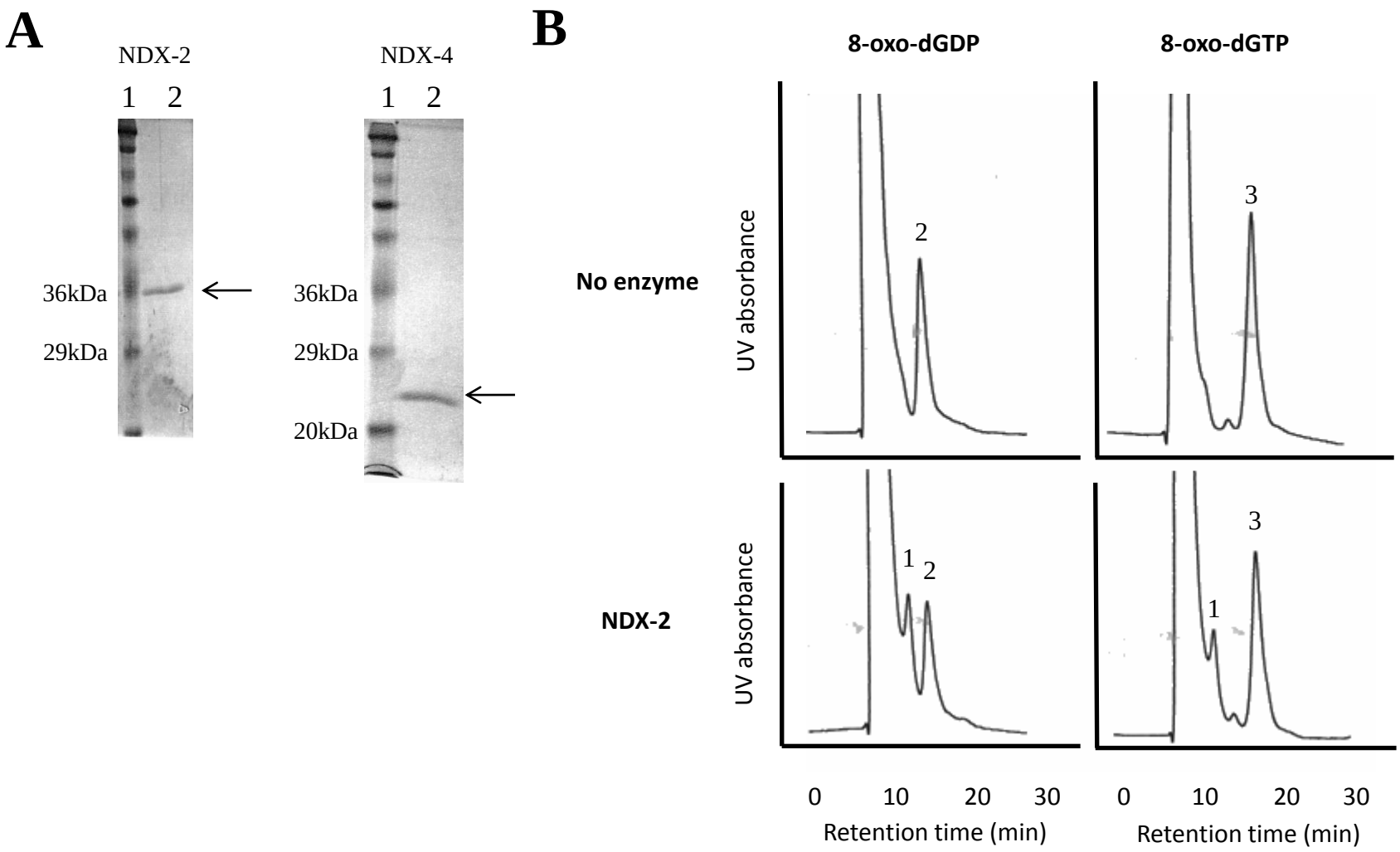

A
NDX-2
1 2
36kDa
29kDa
NDX-4
1 2
36kDa
29kDa
20kDa
B
8-oxo-dGDP
8-oxo-dGTP
No enzyme
NDX-2
UV absorbance
UV absorbance
2
3
1 2
1
3
0 10 20 30
Retention time (min)
0 10 20 30
Retention time (min)

Figure 1

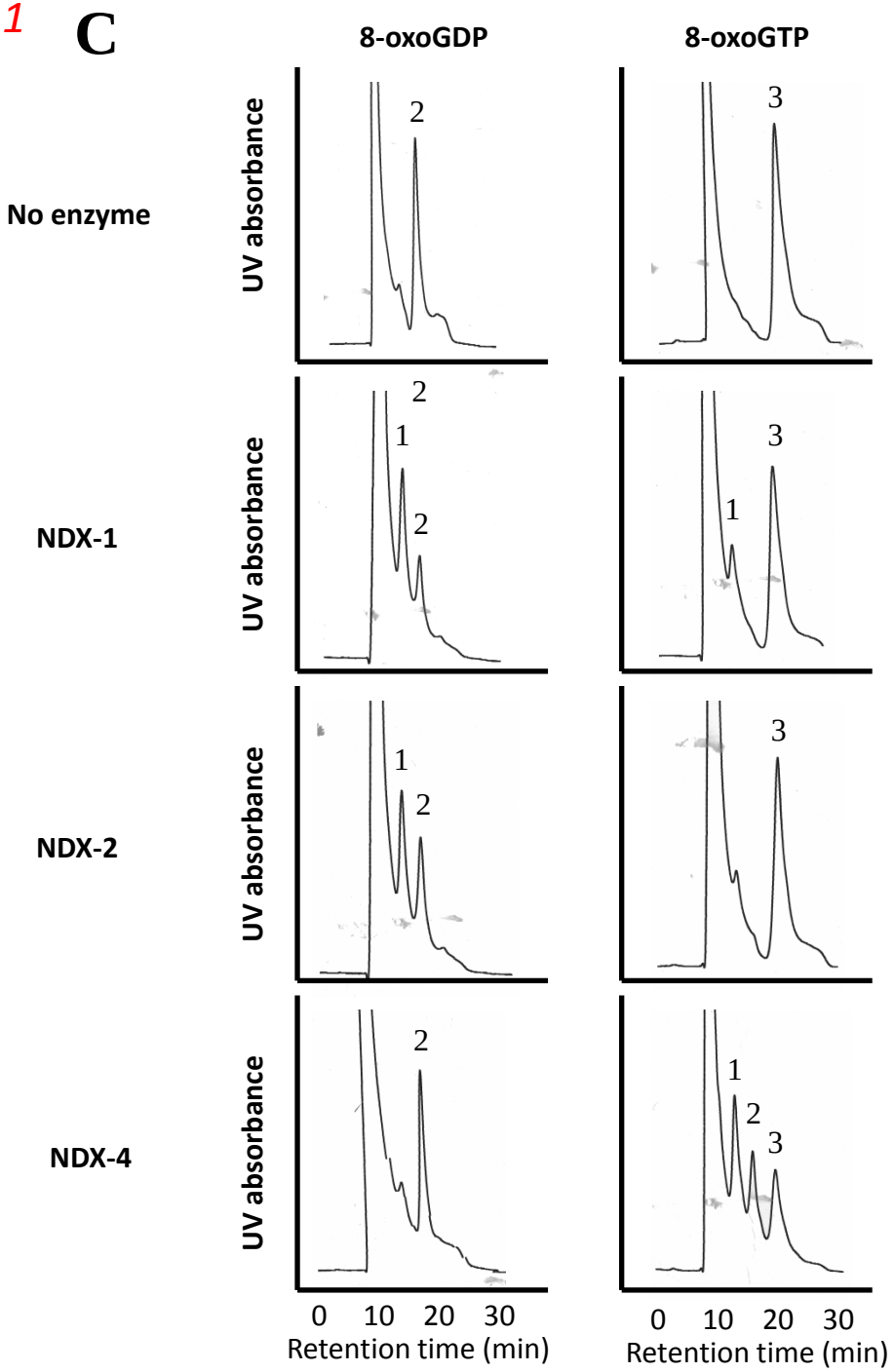

C
8-oxoGDP
8-oxoGTP
No enzyme
NDX-1
NDX-2
NDX-4
UV absorbance
2
3
1
0 10 20 30
Retention time (min)

Figure 2

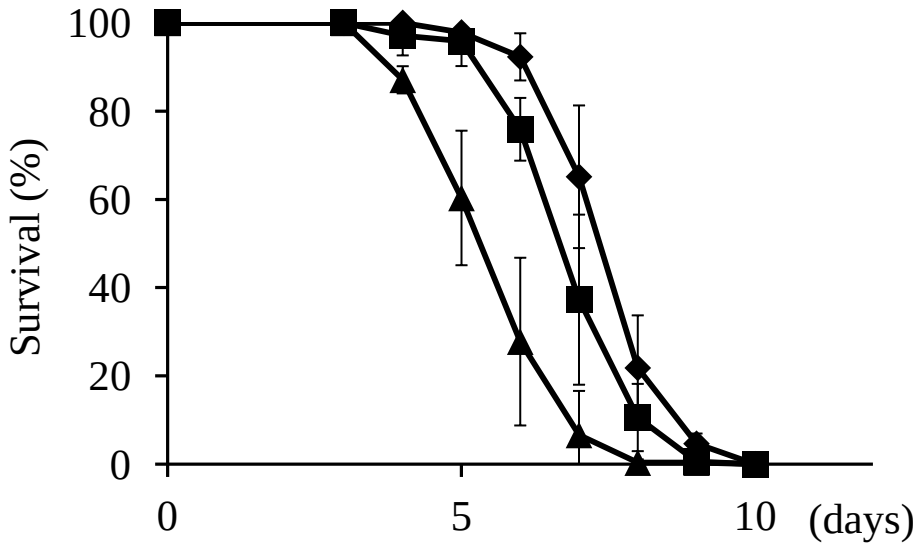


Figure 3

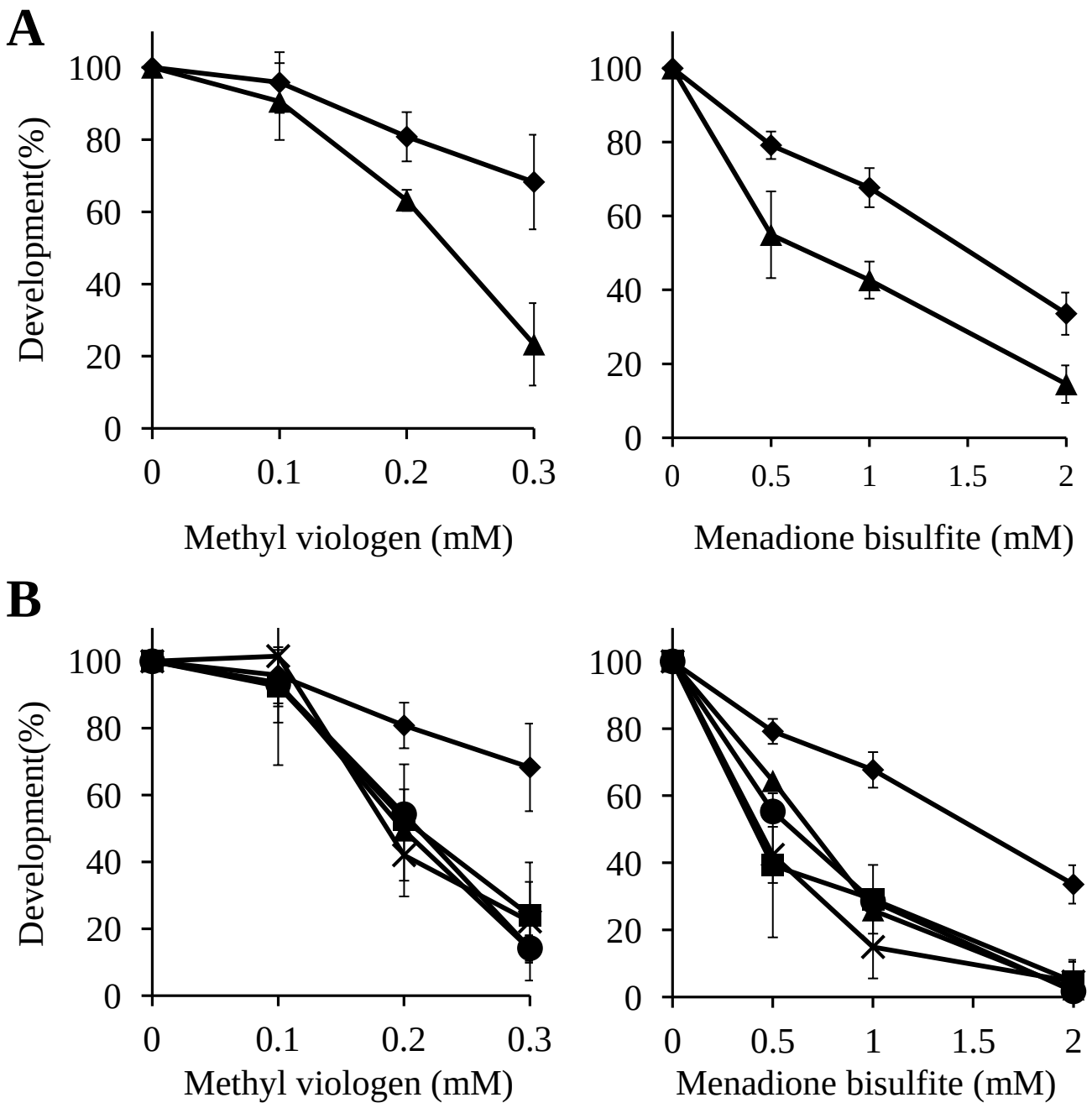

A
Development(%)
100
80
60
40
20
0
0
0.1
0.2
0.3
Methyl viologen (mM)
0
0.5
1
1.5
2
Menadione bisulfite (mM)
B
Development(%)
Methyl viologen (mM)
Menadione bisulfite (mM)

Figure 4

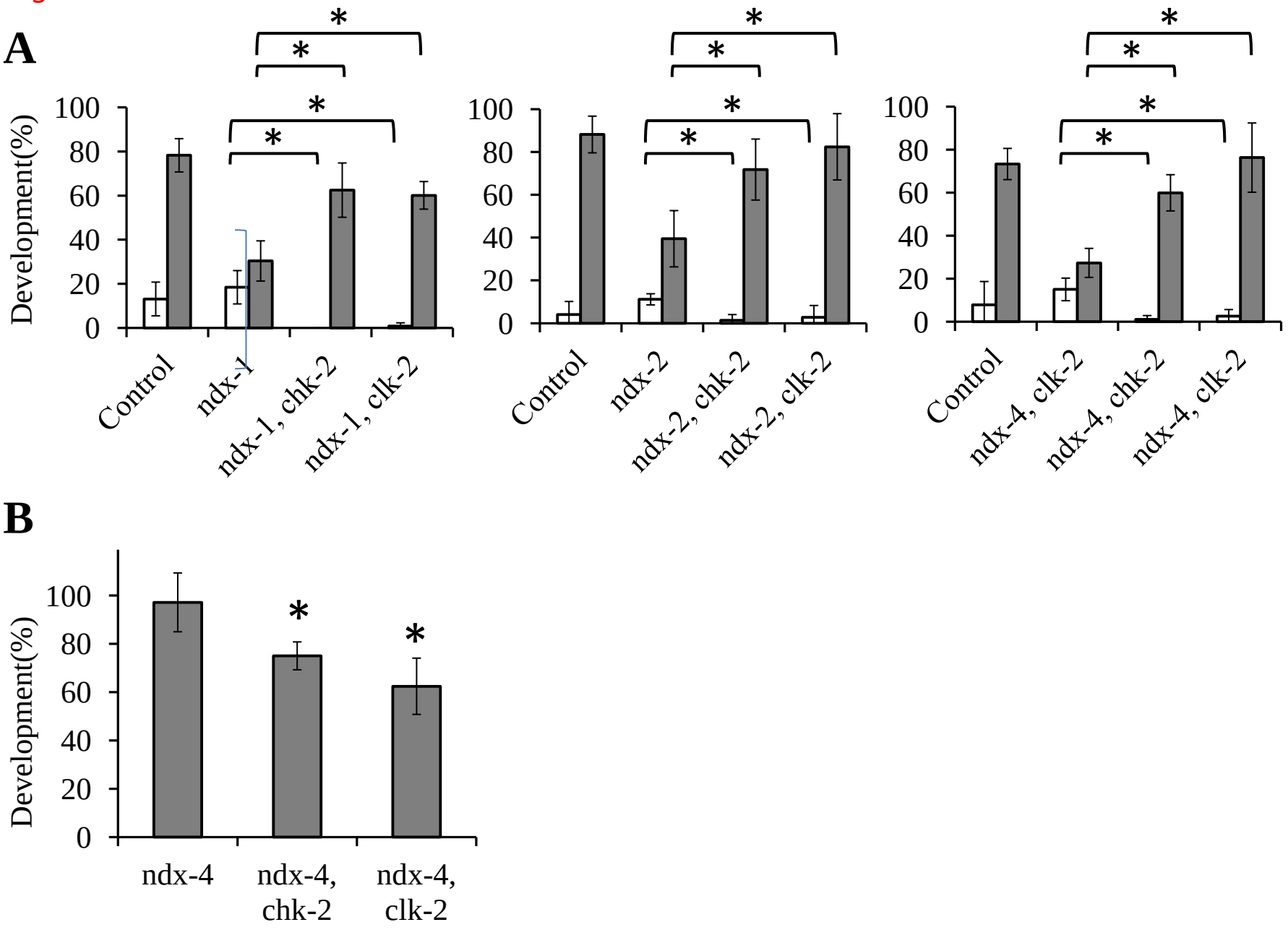
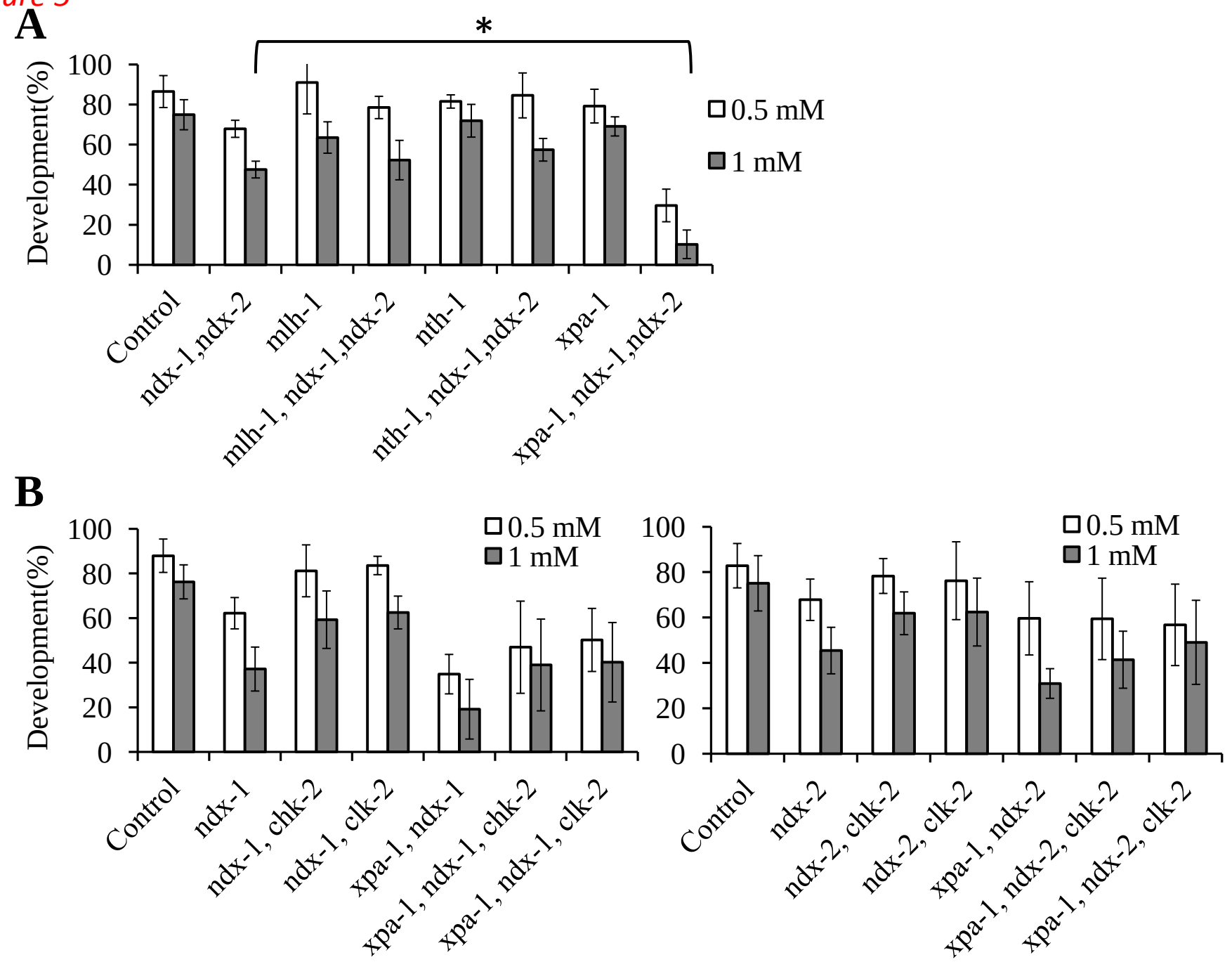
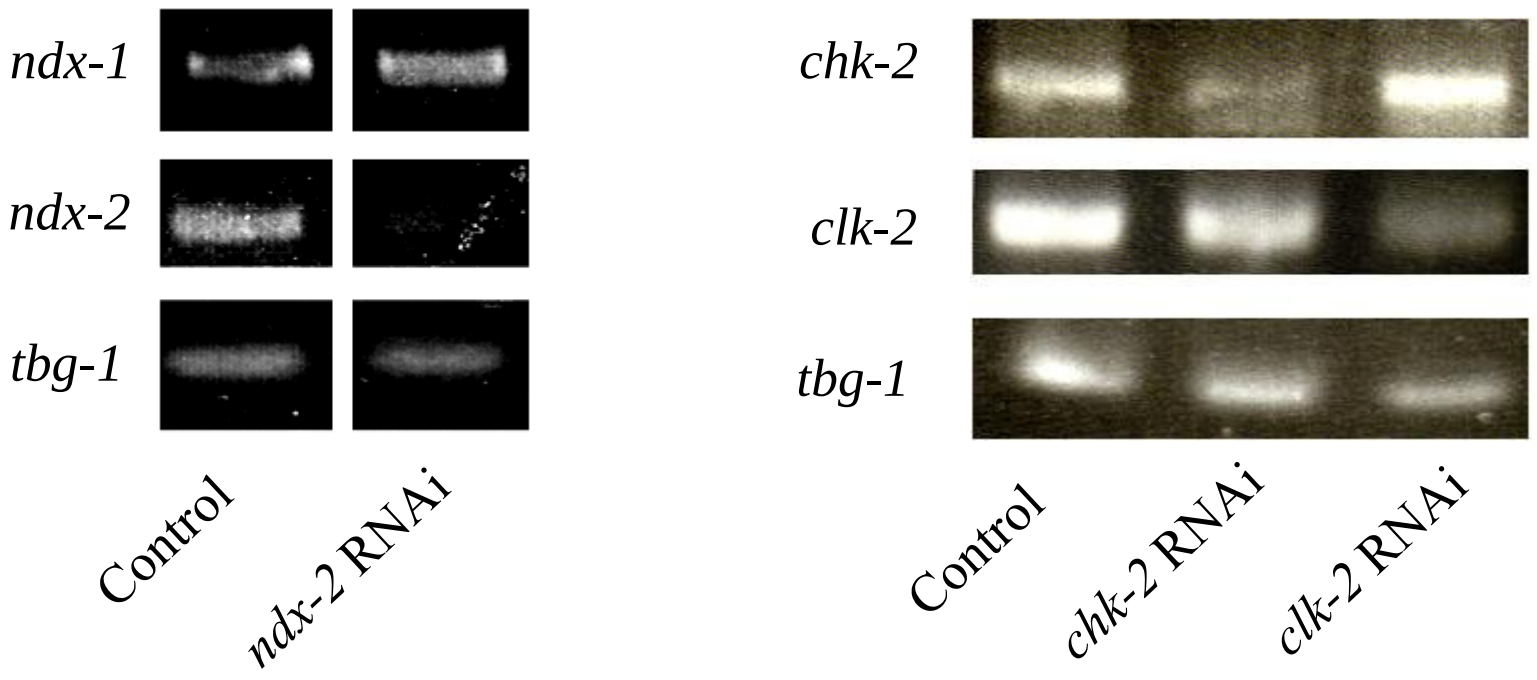
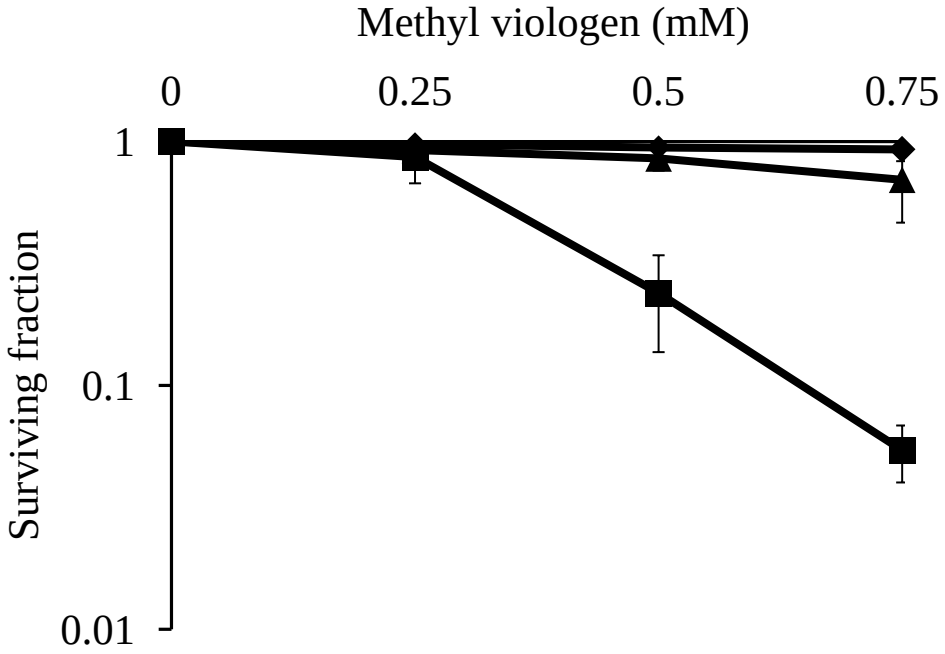

A
Development(%)
100
80
60
40
20
0
*
Control
ndx-1
ndx-1, chk-2
ndx-1, clk-2
ndx-2
ndx-2, chk-2
ndx-2, clk-2
ndx-4, clk-2
ndx-4, chk-2
ndx-4, clk-2
B
ndx-4
ndx-4, chk-2
ndx-4, clk-2

Figure 5

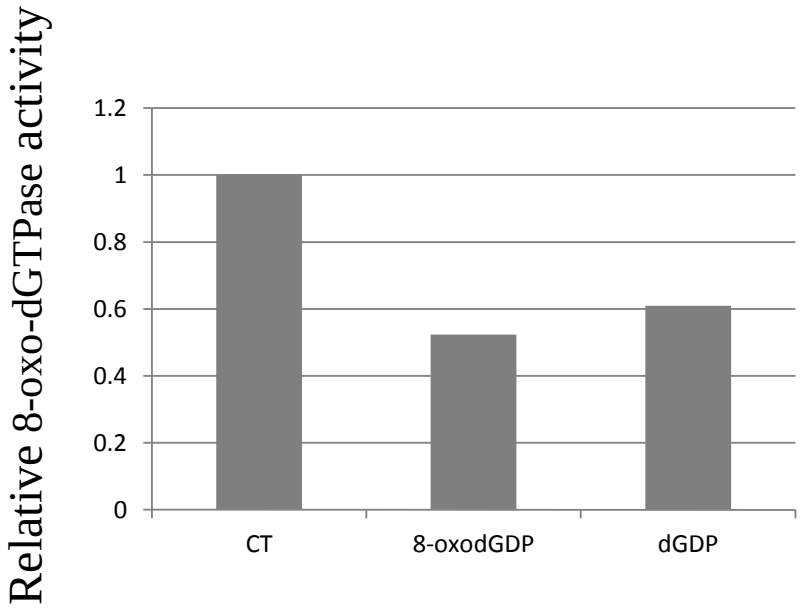

A
*
Development(%)
100
80
60
40
20
0
0.5 mM
1 mM
Control
ndx-1,ndx-2
mlh-1
mlh-1, ndx-1,ndx-2
nth-1
nth-1, ndx-1,ndx-2
xpa-1
xpa-1, ndx-1,ndx-2
B
Development(%)
0.5 mM
1 mM
Control
ndx-1
ndx-1, chk-2
ndx-1, clk-2
xpa-1, ndx-1
xpa-1, ndx-1, chk-2
xpa-1, ndx-1, clk-2
Control
ndx-2
ndx-2, chk-2
ndx-2, clk-2
xpa-1, ndx-2
xpa-1, ndx-2, chk-2
xpa-1, ndx-2, clk-2



Supplementary figure 1. The effect of RNAi. RT-PCR of total RNA isolated from *ndx-1*(RNAi)-, *ndx-2*(RNAi), *chk-2*(RNAi), *clk-2*(RNAi) and control worms. *tbg-1* was used as an internal standard.



Supplementary figure 2. Complementation assay. The sensitivity of *E. coli* CC101, CC101 *mutT* and CC101 *mutT* expressing NDX-2 to Methyl viologen (MV). The overnight cultures were plated on LB agar plates containing MV and the number of colonies was counted to estimate survival after 20 hr of incubation at 37°C. The values represent the mean ± standard deviation (n=4). ◆, *E. coli* CC101; ■, *E. coli* CC101 *mutT*; ▲, *E. coli* CC101 *mutT* expressing NDX-2.



Supplementary figure 3. Inhibition of 8-oxo-dGTPase activity of NDX-4 by 8-oxo-dGDP and dGDP. 8-oxo-dGTP was incubated at 37°C for 30 min with XXX µM of purified NDX-4 in the presence of 8-oxo-dGDP or dGDP.