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# Human NTH1 physically interacts with p53 and proliferating cell nuclear antigen

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**Abstract**

Thymine glycol (Tg) is one of predominant oxidative DNA lesions caused by ionizing radiation and other oxidative stresses. Human NTH1 is a bifunctional enzyme with DNA glycosylase and AP lyase activities and removes Tg as the first step of base excision repair (BER). We have searched for the factors interacting with NTH1 by using a pull-down assay and found that GST-NTH1 fusion protein precipitates proliferating cell nuclear antigen (PCNA) and p53 as well as XPG from human cell-free extracts. GST-NTH1 also bound to recombinant FLAG-tagged XPG, PCNA, and $(His)_6$-tagged p53 proteins, indicating direct protein–protein interaction between those proteins. Furthermore, His-p53 and FLAG-XPG, but not PCNA, stimulated the Tg DNA glycosylase/AP lyase activity of GST-NTH1 or NTH1. These results provide an insight into the positive regulation of BER reaction and also suggest a possible linkage between BER of Tg and other cellular mechanisms.


*Keywords:* Thymine glycol; Base excision repair; NTH1; p53; PCNA; XPG

Oxidative phosphorylation in mitochondrial electron transport chain is essential for eukaryotes to produce energy for maintaining life. It is inevitable, however, that this energy system generates reactive oxygen species (ROS) such as superoxide, hydroxy peroxide, and hydroxyl radical as by-products. ROS is also produced by exposure to chemical oxidant or ionizing radiation [1,2]. The ROS is so highly reactive to oxidize lipids, proteins, and nucleic acids [1–4]. Especially, oxidative DNA damage is thought to cause mutation and genomic instability, possibly leading to cancer and aging [1,3–5].

Thymine glycol (Tg; 5,6-dihydroxy-5,6-dihydrothymine) is one of the major oxidative DNA lesions caused by ionizing radiation and other oxidative stresses such as $H_2O_2$ [2,5–7]. Tg blocks DNA replication, presumably due to the large distortion of DNA double helix [8]. Living organisms have evolved various defense systems against the oxidative DNA damage [1,2,6]. Base excision repair (BER) is the major repair pathway for the oxidized DNA bases and is generally initiated by DNA glycosylases that cut a glycoside bond between the modified base and sugar [2,6,9,10]. DNA glycosylase against Tg in *Escherichia coli* is endonuclease III (endoIII) which is the *nth* gene product and bifunctional enzyme with DNA glycosylase and AP lyase activities [11,12]. The endoIII also excises other modified pyrimidine residues including 5,6-dihydrothymine, 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrouracil (DHU), 5-hydroxy-5,6-dihydrouracil, uracil glycol, and urea [13].

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Human endoIII homolog NTH1 has been identified and characterized for the enzymatic activity of DNA glycosylase and AP lyase against Tg and DHU [14,15]. To complete the BER reaction initiated by NTH1, the sugar moiety at the 3′ end is removed by AP endonuclease 1 (APE1 also called APEX1, HAP1 or Ref1) and the resultant gap is processed in two alternative pathways, a short patch pathway and a long patch pathway [6,9]. The short patch BER is conducted by DNA polymerase β (Pol β) and DNA ligase III/XRCC1 heterodimer or DNA ligase I, and inserts only 1 nucleotide. On the other hand, the long patch BER pathway produces a repair patch of 2–6 nucleotides and can be reconstituted in vitro with replication factor C, proliferating cell nuclear antigen (PCNA), flap endonuclease 1 (FEN1), DNA polymerases δ or ε, and DNA ligase I.

Recently, several factors have been reported to stimulate NTH1 activity. Two independent studies showed that nucleotide excision repair (NER) factor, XPG, stimulates NTH1 activity by enhancing the NTH1 binding to Tg-containing DNA [16,17]. NER is another repair system for removing ultraviolet (UV)-induced cyclobutane pyrimidine dimers and (6–4) photoproducts or bulky chemical adducts [2,18,19]. XPG is a structure-specific endonuclease and makes a nick on the 3′ side to the lesion in the dual incisions of NER [20–22]. Human damage-inducible transcription factor, Y-box binding protein 1 (YB-1 also called DBPB or NSEP), stimulates the NTH1-catalyzed β-elimination reaction [23,24]. APE1 is also known to stimulate the same reaction and the dissociation of NTH1 from the AP (apurinic/apyrimidinic) site [24].

The association between BER and DNA replication has been suggested from several groups. UNG2, a nuclear form of human uracil DNA glycosylase, interacts with PCNA and replication protein A (RPA) and colocalizes with both factors in replication foci, suggesting the coupling of BER and DNA replication to remove misincorporated uracils [25]. Furthermore, MYH, which removes adenine misincorporated opposite 8-oxoG (7,8-dihydro-8-oxoguanine) in template, physically interacts with PCNA, RPA, and APE1 [26], and colocalizes with PCNA in replication foci [27]. It is likely that PCNA plays important roles in post-replicative repair (replication-coupled repair) through the protein–protein interaction.

p53 has been also shown to associate with BER. p53 is well known as a tumor suppressor and a gatekeeper for maintaining the genomic integrity. The cellular level and activity of p53 are down-regulated under the physiological condition by a ubiquitin–proteasome pathway. Various stress signals including oxidative DNA damage induce the post-translational accumulation of p53 and enhance its transactivation activity, leading to the up-regulation of down-stream genes involved in cell cycle arrest, DNA repair, and apoptosis [28–30]. Interestingly, p53 directly interacts with Pol β and APE1, and stabilizes the binding of Pol β to abasic DNA, resulting in the stimulation of BER for uracil and natural AP site [31].

In order to understand the molecular mechanisms for the efficient repair of Tg lesions, we have searched for the factors that interact with NTH1 and modulate its activity. We report here that NTH1 physically interacts with PCNA and p53 as well as XPG. Furthermore, we show that p53 and XPG but not PCNA stimulate the Tg DNA glycosylase/AP lyase activity of NTH1.

## Materials and methods

*Preparation of recombinant NTH1 protein*. Human NTH1 cDNA was obtained by RT-PCR (GenBank XM_028895). Total RNA was prepared from WI26VA4 cells (normal human fibroblast cell line) using RNeasy Mini Kit (Qiagen) and the first-strand cDNA was generated with SuperScript II RNase$^-$ reverse transcriptase (Invitrogen). The NTH1 open reading frame was divided into three parts which are partially overlapping, and the following primers were designed to amplify each cDNA fragment: 1–406, F1 (5′-TGGGATCC ATGTGTAGTCCGCAGGAGTC-3′) and R1 (5′-TGGAATTCGTG ACAGCAGCACCTGGTA-3′); 373–831, F2 (5′-TGGGATCCCCA AAGGTACGCAGGTACCA-3′) and R2 (5′-TGGAATTCCTCGTG CCACAGCTCCCTA-3′); and 797–1007, F3 (5′-TGGGATCCTGGA GGAGTGGCTGCCTA-3′) and R3 (5′-TGGAATTCTTCCTGAAG CGTAAAGCCAC-3′). The PCR was conducted (1 cycle at 94 °C for 1 min; 30–35 cycles at 94 °C for 30 s, 66 or 64 °C for 1 min, and 72 °C for 2 min; and 1 cycle at 72 °C for 10 min) using *pyrobest* DNA polymerase (Takara). The products were digested with *Bam*HI and *Eco*RI, and individually subcloned into the pGEX-4T-1 vector (Amersham Biosciences). An expression construct for full length NTH1 fused with glutathione *S*-transferase (GST) was obtained by reconstructing the three plasmids and verified by sequencing with Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit and Long-Read Tower DNA sequencer (Amersham Biosciences).

The GST-NTH1 protein was overproduced in *E. coli* BL21 (DE3)pLys cells (Novagen) by incubating with 0.1 mM IPTG for 3 h at 30 °C. Cells were resuspended in 40 ml of sonication buffer (50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 0.02% Triton X-100, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin) and sonicated on ice using Microson ultrasonic cell disruptor model XL 2000 (Misonix). After centrifugation, the supernatant was applied to a glutathione–Sepharose 4B (Amersham Biosciences) column (1 ml) and the GST fusion proteins were eluted with sonication buffer containing 15 mM reduced glutathione (Sigma). The peak fractions were dialyzed against dialysis/storage buffer (25 mM Hepes–KOH (pH 7.9), 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, and 16–17% glycerol) and stored at −80 °C.

GST-free NTH1 protein was generated by digestion of GST-NTH1 using Thrombin cleancleave kit (Sigma). GST-NTH1 was incubated for 6 h at 4 °C with thrombin-agarose equilibrated with dialysis/storage buffer, and GST and undigested fusion proteins were eliminated by centrifugation with glutathione–Sepharose 4B.

*Preparation of FLAG-tagged XPG protein in the baculovirus/insect cell system*. To overproduce FLAG-tagged XPG using the Bac-to-Bac baculovirus expression system (Invitrogen), human XPG cDNA was subcloned into the pFASTBac1 expression vector. The 5′-terminal portions of cDNAs were amplified by PCR using the primers with FLAG epitope sequences (underlined) and *Spe*I restriction site (small letters), 5′-cggactagtATGGACTACAAAGACGATGACGACAAGA TGGGGGTCCAGGGGCTC-3′ and 5′-TGTTTTCAGAAGCTTTC TGTCG-3′. The product was digested with *Spe*I and *Hin*dIII,

subcloned into the pFASTBac1-XPG, and verified by sequencing. Recombinant baculovirus encoding the FLAG-XPG was prepared according to the manufacturer's protocol (Invitrogen) and infected into *Sf*21 insect cells at 27 °C for ~48 h. The cells were collected and resuspended in 10 volumes of lysis buffer A (20 mM Tris–HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin). After incubation on ice for 15 min, the cleared cell lysate was mixed with 1-ml bed volume of anti-FLAG M2 affinity gel (Sigma) and gently agitated at 4 °C for 1 h. After washing with buffer A (25 mM Hepes–KOH (pH 7.9), 150 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and 10% glycerol), the FLAG-tagged XPG protein was eluted with buffer A containing 100 μg/ml FLAG peptide (Sigma). The peak fractions were dialyzed against dialysis/storage buffer and stored at −80 °C.

*Preparation of (His)$_6$-tagged p53 protein*. Recombinant baculovirus encoding (His)$_6$-tagged p53 was kindly obtained from Dr. Hideyo Yasuda (Tokyo University of Pharmacy and Life Science). *Sf*21 insect cells were infected with the virus and incubated at 27 °C for ~48 h. The cell lysate was prepared as described above and imidazole was added at a final concentration of 20 mM. The protein solution was mixed with 3-ml bed volume of Ni–NTA agarose (Qiagen) and incubated at 4 °C for 2 h with gentle agitation. After extensive washing with buffer B (20 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and 1 mM DTT) containing 20 mM imidazole, the His-p53 protein was eluted with buffer B containing 50 or 100 mM imidazole. The peak fractions were pooled, dialyzed against dialysis/storage buffer, and stored at −80 °C.

*Cell culture*. WI38VA13 (SV40-transformed normal human cell line), XPCS2LV(SV) (SV40-transformed XP-G/CS cell line), and A549 (human lung adenocarcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 50 μg/ml gentamicin at 37 °C in a 5% $CO_2$ atmosphere. XPCS2LV(SV) (GM14930) and A549 (JCRB0076) were obtained from the Coriell Institute for Medical Research (NJ, USA) and the Health Science Research Resources Bank (Osaka, Japan), respectively. *Sf*21 insect cells were purchased from Invitrogen and cultured in Grace's Insect medium containing 10% FBS at 27 °C.

*Preparation of cell lysate after $H_2O_2$ treatment*. Cultured A549 cells were washed with PBS (−) twice and treated with or without $H_2O_2$ in DMEM at 37 °C for 5 min. The cells were washed with PBS (−) twice and incubated in the culture medium for 0, 2 or 4 h at 37 °C in a 5% $CO_2$ atmosphere. After washing with PBS (−), lysis buffer B (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin) was added to the cells and incubated on ice for 30 min. The cell lysate was centrifuged at 2 °C for 15 min and the supernatant was stored at −80 °C.

*Pull-down assay*. Cell-free extracts (CFEs) were prepared by the method of Manley et al. [32] and recombinant human PCNA was prepared as described previously [33]. GST-NTH1 was incubated with CFEs or recombinant proteins (FLAG-XPG, PCNA or His-p53) at 4 °C overnight in 200 μl of pull-down buffer A (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% or 1% Nonidet P-40, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin) or pull-down buffer B (25 mM Hepes–KOH (pH 7.9), 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, and 1% Nonidet P-40), and subsequently mixed with glutathione–Sepharose 4B beads at 4 °C for 1.5 h. After centrifugation at 2 °C for 1 min, the pellet was washed three times with 1 ml of pull-down buffer A or B and boiled in Laemmli sample buffer (Bio-Rad). The bound proteins were analyzed by Western blotting and detected with NBT/BCIP (Promega) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and lumino-image analyzer LAS1000 (Fuji film). Monoclonal antibody (G-26) specific for human XPG was obtained by immunizing mice with the fusion protein of XPG and maltose-binding protein. Mouse anti-p53 antibody was purchased from Santa Cruz Biotechnology and mouse anti-FLAG M2 was obtained from Sigma. Mouse anti-PCNA and anti-RPA(p34) antibodies were from Calbiochem and rabbit anti-NTH1 antibody was from Alpha Diagnostic.

*Tg DNA incision assay*. A 45-bp duplex DNA substrate containing a single thymine glycol (Tg) was assembled from four oligonucleotides (IwTg, ACGCGAtACGCCA; Tg left, CGACCATGCCTGCACG AA; Tg right, GTGCAGGCAGGTCA; and Tg bottom; TATGA CCTGCCTGCACTGGCGTATCGCGTTTCGTGCAGGCATGGT) (Fig. 1A). These oligonucleotides were kindly provided by Drs. Shigenori Iwai (Osaka University) and Akira Yasui (Tohoku University). The oligonucleotide with Tg was labeled with [$\gamma$-$^{32}$P]ATP (NEN Life Science Product) and T4 polynucleotide kinase (New England Biolabs). After annealing with other three oligonucleotides, T4 DNA ligase (Invitrogen) was added to the mixture for constructing a full length DNA substrate.

The substrate DNA (1 fmol) was incubated with GST-NTH1 or NTH1 in 10 μl of reaction buffer (25 mM Tris–HCl (pH 8.0), 2 mM EDTA, and 70 mM NaCl) at 30 °C for the indicated periods, and 10 μl of gel-loading buffer (10 mM EDTA, 0.1% xylene cyanol FF, and 0.1% bromophenol blue in formamide) was added to stop the reaction. After heat denaturation at 95 °C for 10 min, the reaction mixture was separated on 10% denaturing polyacrylamide gels and visualized by autoradiography or quantified with a Bas 2000 Bioimaging Analyzer (Fuji film).

## Results and discussion

### *Preparation of functionally active GST-NTH1*

In order to identify the cellular factors that interact with NTH1 and stimulate its activity, recombinant GST-NTH1 protein was overproduced in *E. coli* and purified using glutathione–Sepharose affinity beads. The purified GST-NTH1 was tested for DNA glycosylase/AP lyase activity by incubating with $^{32}$P-labeled DNA substrate containing a single Tg (Fig. 1A). As shown in Fig. 1B, incision products increased depending on the reaction time and the amount of GST-NTH1. GST alone also showed a weak signal, but the level is comparable to the control reaction with no protein. These data indicate that the purified recombinant GST-NTH1 is functionally active.

### *Coprecipitation of XPG, PCNA, and p53, but not RPA, with GST-NTH1 from human cell-free extracts*

One of NER factors, XPG, has been shown to stimulate NTH1 activity, although the physical interaction of the two proteins is still not clear [16,17]. We decided to test whether NTH1 associates with XPG using a pull-down assay, in which CFEs from WI38VA13 or XPCS2LV(SV) cells were incubated with GST-NTH1. Immunoblot analysis of the bound fractions revealed that XPG was coprecipitated with GST-NTH1, but not GST, suggesting the association between NTH1 and XPG (Fig. 2A).

For the survey of NTH1-interacting proteins, the bound fractions in the pull-down experiments were screened using various antibodies as probes. We first selected the antibodies specific for PCNA and RPA, which

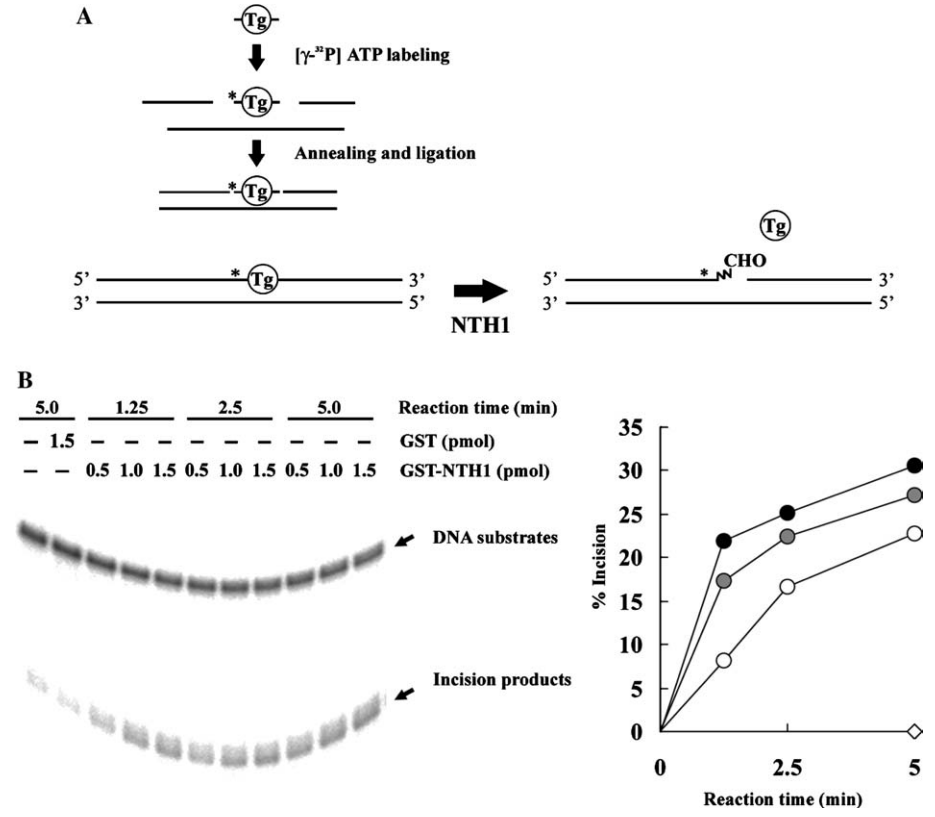


Fig. 1. GST-NTH1 is functionally active in a Tg DNA incision assay. (A) Construction of internally $^{32}$P-labeled duplex DNA substrate containing a single thymine glycol (Tg). The substrate was prepared by phosphorylation of Tg-containing oligonucleotide with [γ-$^{32}$P]ATP and subsequent ligation with other three overlapping oligonucleotides. Asterisks denote the position of $^{32}$P-radiolabel and Tg locates at the 25th position from the 5′ end. (B) Tg DNA glycosylase/AP lyase activity of purified recombinant GST-NTH1. (Left) $^{32}$P-labeled DNA substrate containing a single Tg (1 fmol) was incubated with or without the indicated amounts of GST-NTH1 or GST. The products were separated on a 10% denaturing polyacrylamide gel and visualized by autoradiography. (Right) Quantitative analysis of the data shown in the left panel. Symbols: open circles, 0.5 pmol GST-NTH1; gray circles, 1.0 pmol GST-NTH1; black circles, 1.5 pmol GST-NTH1; and open diamond, 1.5 pmol GST. The value of background (no protein) was subtracted from each value of samples.

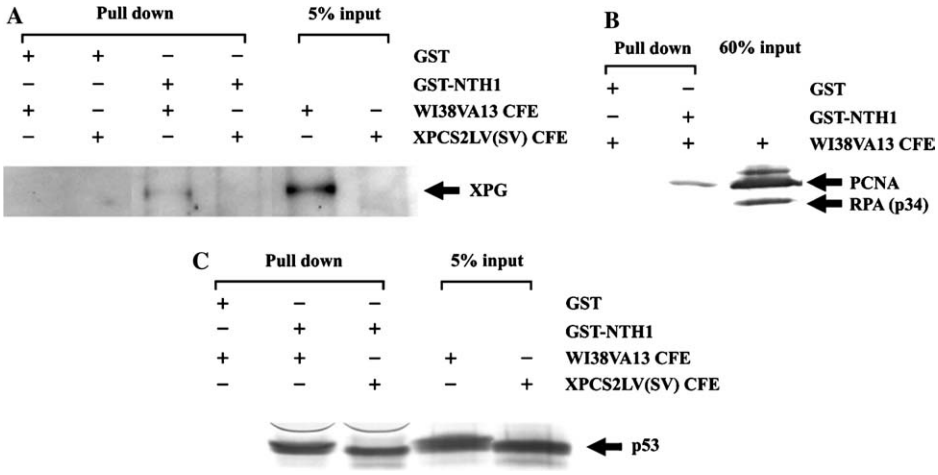


Fig. 2. XPG, PCNA, and p53, but not RPA, were coprecipitated with GST-NTH1 from human cell-free extracts. Fifty micrograms of GST-NTH1 or 22.7 μg GST was incubated with CFEs from the human SV40-transformed cell lines and the bound fractions were analyzed by Western blotting with various antibodies. (A) GST-NTH1 binds to XPG in CFEs. Four hundred micrograms of WI38VA13 (normal) or XPCS2LV(SV) (XP-G/CS) CFEs was subjected to the pull-down experiment and anti-XPG antibody was used as a probe. (B) GST-NTH1 binds to PCNA but not RPA in CFEs. One hundred micrograms of WI38VA13 CFEs was pulled down and anti-PCNA and anti-RPA(p34) antibodies were used as probes. (C) GST-NTH1 binds to p53 in CFEs. One hundred micrograms of WI38VA13 or XPCS2LV(SV) CFEs was pulled down and anti-p53 antibody was used as a probe.

are known to link with BER [6,25–27]. We found that PCNA, but not RPA, is specifically detected in the bound fractions with GST-NTH1 (Fig. 2B), suggesting that NTH1 associates with PCNA.

We next asked whether NTH1 interacts with p53, since the p53 has been suggested to play direct roles in BER by interacting with Pol β and/or APE1 [31]. Anti-p53 antibody was used as a probe after pull-down experiments and revealed specific signals in the bound fraction with GST-NTH1 (Fig. 2C). In the SV40-transformed cell lines, however, p53 is known to accumulate abnormally due to the expression of large T antigen [34]. We decided to conduct similar experiments using A549 lung adenocarcinoma cell line with normal p53 response. Indeed, the basal level of p53 in this cell line was much lower than that in WI38VA13 cells (Fig. 3A), and $H_2O_2$ treatment induced p53 accumulation (Fig. 3B) [35]. CFEs from A549 cells treated with or without $H_2O_2$ were prepared and mixed with GST or GST-NTH1. Again, p53 was detected in the GST-NTH1-bound fractions, and larger amounts of p53 were coprecipitated from the CFEs prepared from $H_2O_2$-treated cells, apparently corresponding to the cellular p53 level (Fig. 3B). We could also detect endogenous p53 in the bound fractions when cell lysates from A549 stably overexpressing NTH1-3×FLAG were immunoprecipitated with anti-FLAG antibody (data not shown).

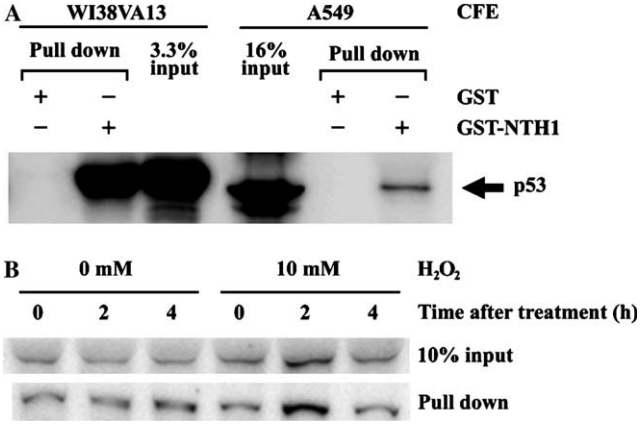


Fig. 3. $H_2O_2$ treatment increased the cellular level of p53 in A549 cells and the accumulated p53 was coprecipitated with GST-NTH1. (A) GST-NTH1 binds to p53 in CFEs from A549 cell line with normal p53 response. Fifty micrograms of GST-NTH1 or 22.7 μg GST was incubated with 100 μg WI38VA13 CFEs or 200 μg of A549 CFEs and the bound fractions were analyzed by Western blotting with anti-p53 antibody. (B) p53 accumulates in A549 cells after treatment of $H_2O_2$ and correspondingly binds to GST-NTH1. A549 cells were treated with or without 10 mM $H_2O_2$ at 37 °C for 5 min, incubated for the indicated periods after medium change, and processed for cell lysate preparation. Fifty micrograms of GST-NTH1 was incubated with 100 μg of the cell lysate, and the bound fractions were analyzed with anti-p53 antibody.

*NTH1 directly interacts with recombinant XPG, PCNA, and p53*

Having shown that NTH1 associates with XPG, PCNA, and p53 in human CFEs, we wished to know whether the association is direct or indirect. Recombinant FLAG-tagged XPG, PCNA, and $(His)_6$-tagged p53 proteins were prepared and employed for the pull-down experiments with GST-NTH1 or GST alone. As shown in Figs. 4A and B, the recombinant FLAG-XPG and PCNA were found to bind to GST-NTH1 with a high specificity. The recombinant His-p53 was also coprecipitated with GST-NTH1, but background precipitation could be detected although with less efficiency (Fig. 5A). We further carried out the "reverse" pull-down experiments in which GST-p53 purified from the baculovirus/insect cell system was mixed with NTH1 prepared from GST-NTH1 after digestion with thrombin (Fig. 5B). We confirmed the specific interaction between the two proteins and concluded that NTH1 physically interacts with PCNA and p53 as well as XPG. The PIP-box and KA-box are well known as PCNA-binding motifs [36]. However, we could not find those motifs in the NTH1 sequence. Several proteins such as GADD45, MyD118, and CR6 bind to PCNA without the PCNA-binding motifs [36]. NTH1 might be the case and a novel PCNA-interacting domain needs to be identified.

*NTH1 activity is stimulated by XPG and p53 but not PCNA*

The newly identified physical interactions between NTH1 and PCNA or p53 prompted us to examine their effects on NTH1 activity. We conducted a Tg DNA incision assay in the presence or absence of recombinant FLAG-XPG, PCNA or His-p53. As shown in Fig. 4C, FLAG-XPG stimulated the NTH1 activity in a dose-dependent manner, consistent with the previous observations [16,17]. However, PCNA failed to enhance the incision activity of GST-NTH1, even when 4-fold excess molar PCNA was used (Fig. 4D). On the other hand, His-p53 was also found to stimulate the NTH1 activity dose-dependently (Fig. 5C). It is noteworthy that equimolar XPG or p53 to NTH1 is capable of stimulating the NTH1 activity. It has been reported that GST-fused NTH1 is less active compared with GST-free NTH1 after digestion by thrombin [15]. To exclude the possible effects of GST on the reaction, NTH1 was purified from GST-NTH1 treated with thrombin and used for the Tg DNA incision assay. The NTH1 protein showed higher DNA glycosylase/AP lyase activity than parental GST-NTH1 (data not shown), consistent with the previous report. The activity of the NTH1 preparation was similarly stimulated by His-p53 in a dose-dependent manner (Fig. 5D).

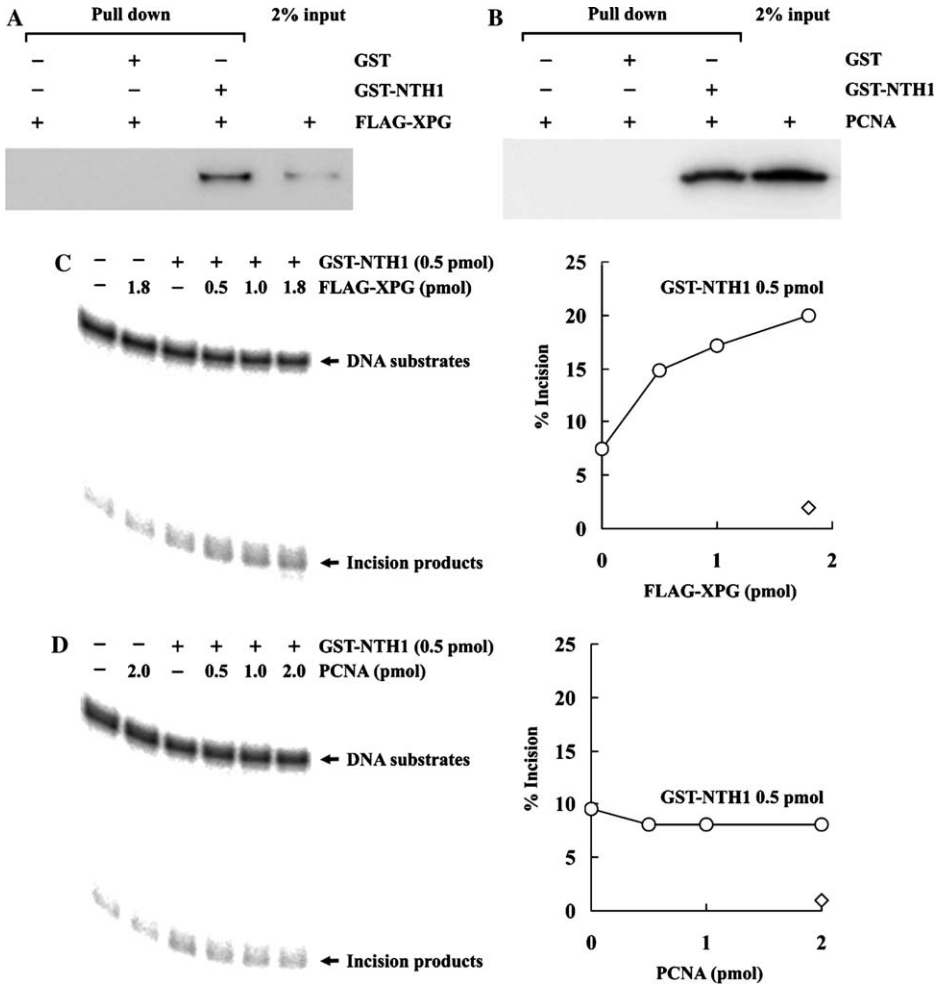


Fig. 4. NTH1 physically interacts with XPG and PCNA, and NTH1 activity is stimulated by XPG but not PCNA. (A) GST-NTH1 directly interacts with recombinant FLAG-XPG. Three micrograms of FLAG-XPG was incubated with 1.4 μg GST-NTH1 or 0.6 μg GST and the bound fractions were analyzed by Western blotting with anti-XPG antibody. (B) GST-NTH1 directly interacts with recombinant PCNA. One microgram of PCNA was incubated with 2.1 μg GST-NTH1 or 1 μg GST and anti-PCNA antibody was used as a probe. Note that equimolar proteins were used for the pull-down assays. (C) FLAG-XPG stimulates the Tg DNA glycosylase/AP lyase activity of GST-NTH1. (Left) $^{32}$P-labeled DNA substrate containing a single Tg (1 fmol) was incubated with 0.5 pmol GST-NTH1 in the presence of FLAG-XPG (0, 0.5, 1.0 or 1.8 pmol) for 1.25 min. The products were separated on a 10% denaturing polyacrylamide gel and visualized by autoradiography. (Right) Quantitative analysis of the data shown in the left panel. The value of background (no protein) was subtracted from each value of samples. Symbols: open circles, FLAG-XPG + GST-NTH1; open diamond, FLAG-XPG alone. (D) PCNA does not stimulate the Tg DNA glycosylase/AP lyase activity of GST-NTH1. The Tg-containing DNA substrate (1 fmol) was incubated with 0.5 pmol GST-NTH1 in the presence of PCNA (0, 0.5, 1.0 or 2.0 pmol) for 1.25 min and analyzed as described in (C). Symbols: open circles, PCNA + GST-NTH1; open diamond, PCNA alone.

How does p53 stimulate NTH1 activity? It has been recently reported that XPC-HR23B interacts with TDG, thymine DNA glycosylase, and stimulates its activity by enhancing the turnover of TDG [37]. We conducted a similar type of experiment using GST-NTH1 and His-p53, but could not observe a significant effect of p53 on the turnover of NTH1 (data not shown). On the other hand, p53 is known to stimulate the excision of 5′-dRp by stabilizing the binding of Pol β to AP site through the direct interaction of p53 with Pol β [31]. AP lyase activity is mechanically similar to dRPase activity of Pol β [38,39] and both NTH1/AP lyase and Pol β/dRPase are known to be rate-limiting reactions for those enzymes [23,40]. Taken together, we suspect that p53 may stimulate AP lyase activity of NTH1 probably leading to a short patch BER pathway. Consistent with this idea, Tg has been reported to be repaired mainly by a single nucleotide BER pathway [41]. Further studies are needed to understand the molecular mechanism of the NTH1 stimulation by p53.

On the other hand, PCNA directly bound to NTH1, but did not stimulate the Tg DNA glycosylase/AP lyase activity of NTH1. These data suggest that PCNA might play a role in coordination of the whole BER reaction or in the link between BER and DNA replication, rather than modulation of NTH1 activity itself. UNG2 and

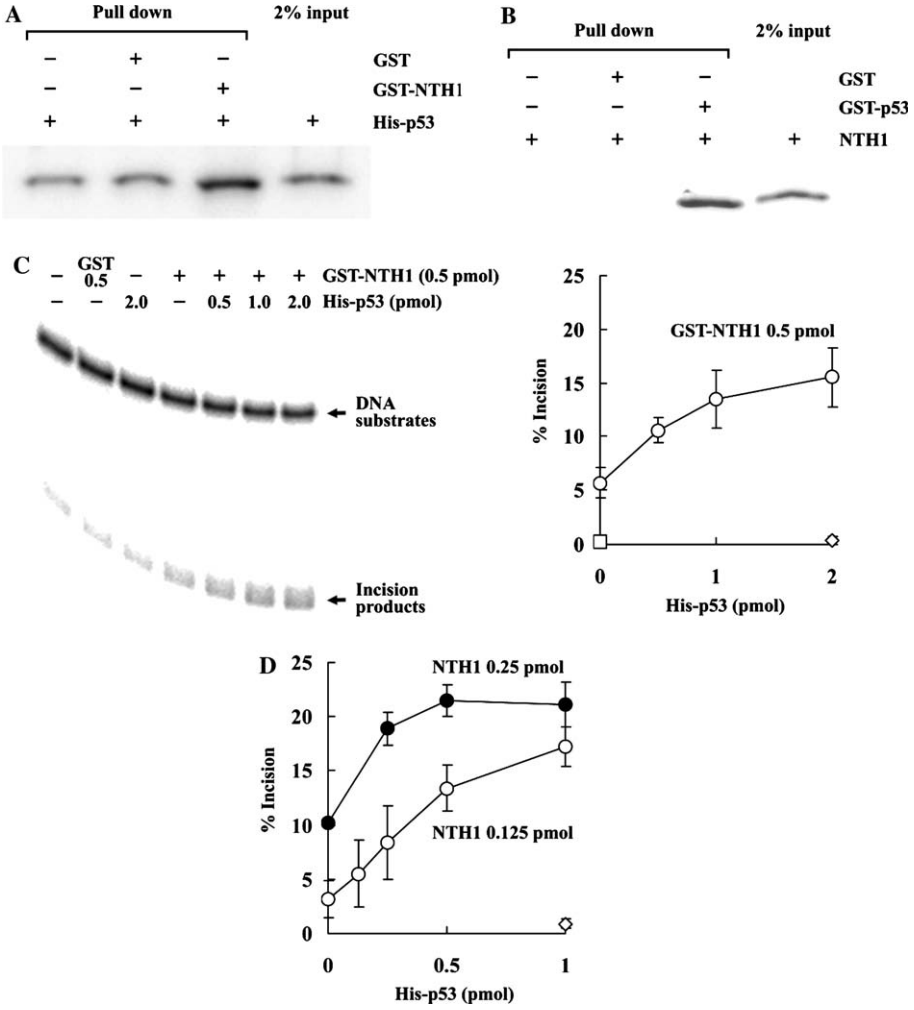


Fig. 5. p53 directly interacts with NTH1 and stimulates its DNA glycosylase/AP lyase activity. (A) GST-NTH1 directly interacts with recombinant His-p53. Three micrograms of His-p53 was incubated with 4.1 μg of GST-NTH1 or 1.9 μg GST and anti-p53 antibody was used as a probe. (B) GST-p53 directly interacts with recombinant NTH1. Ten micrograms of NTH1 prepared from thrombin-digested GST-NTH1 was incubated with 16 μg GST-p53 or 6 μg GST and anti-NTH1 antibody was used as a probe. Note that equimolar proteins were used for the pull-down assay. (C) His-p53 stimulates the Tg DNA glycosylase/AP lyase activity of GST-NTH1. (Left) $^{32}$P-labeled DNA substrate containing a single Tg (1 fmol) was incubated with 0.5 pmol GST-NTH1 in the presence of His-p53 (0, 0.5, 1.0 or 2.0 pmol) for 1.25 min. The products were separated on a 10% denaturing polyacrylamide gel and visualized by autoradiography. (Right) Quantitative data of repeated experiments including the left panel. Bars indicate SD from three independent experiments. Symbols: open circles, His-p53 + GST-NTH1; open diamond, His-p53 alone; and open square, GST alone. (D) His-p53 stimulates the Tg DNA glycosylase/AP lyase activity of GST-free NTH1. The Tg-containing DNA substrate was incubated with 0.125 or 0.25 pmol NTH1 in the presence of His-p53 (0, 0.125, 0.25, 0.5 or 1.0 pmol) for 1.25 min and analyzed as described in (C). Symbols: open and closed circles, His-p53 + NTH1; open diamond, His-p53 alone.

MYH have been shown to interact with PCNA and to be involved in post-replicative BER of misincorporated bases [25–27]. Interestingly, the transcriptional level of NTH1 increases in the early and mid S-phase [42] as seen in UNG2 and MYH [27,43]. PCNA in the stalled replication machinery may recruit NTH1 and other BER factors into Tg lesion through the interactions.

Tg is known to efficiently block DNA replication and transcription. PCNA and XPG may play roles in the coupling of BER with replication and transcription arrests at the Tg sites. Moreover, the interaction between NTH1 and p53 might connect oxidative DNA damage/BER with p53-dependent damage response pathways. Our findings would provide a clue to uncover not only the mechanism of efficient BER but also the association of BER with other cellular systems.

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## References

[1] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing, Nature 408 (2000) 239–247.
[2] E.C. Friedberg, G.C. Walker, W. Siede, DNA Repair and Mutagenesis, American Society for Microbiology Press, Washington, DC, 1995.
[3] B.N. Ames, M.K. Shigenaga, T.M. Hagen, Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. USA 90 (1993) 7915–7922.
[4] M.K. Shigenaga, T.M. Hagen, B.N. Ames, Oxidative damage and mitochondrial decay in aging, Proc. Natl. Acad. Sci. USA 91 (1994) 10771–10778.
[5] R. Adelman, R.L. Saul, B.N. Ames, Oxidative damage to DNA: relation to species metabolic rate and life span, Proc. Natl. Acad. Sci. USA 85 (1988) 2706–2708.
[6] G. Slupphaug, B. Kavli, H.E. Krokan, The interacting pathways for prevention and repair of oxidative DNA damage, Mutat. Res. 531 (2003) 231–251.
[7] X.C. Le, J.Z. Xing, J. Lee, S.A. Leadon, M. Weinfeld, Inducible repair of thymine glycol detected by an ultrasensitive assay for DNA damage, Science 280 (1998) 1066–1069.
[8] J.Y. Kao, I. Goljer, T.A. Phan, P.H. Bolton, Characterization of the effects of a thymine glycol residue on the structure, dynamics, and stability of duplex DNA by NMR, J. Biol. Chem. 268 (1993) 17787–17793.
[9] T. Lindahl, Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair, Mutat. Res. 462 (2000) 129–135.
[10] T. Izumi, L.R. Wiederhold, G. Roy, R. Roy, A. Jaiswal, K.K. Bhakat, S. Mitra, T.K. Hazra, Mammalian DNA base excision repair proteins: their interactions and role in repair of oxidative DNA damage, Toxicology 193 (2003) 43–65.
[11] B. Weiss, R.P. Cunningham, Genetic mapping of nth, a gene affecting endonuclease III (thymine glycol-DNA glycosylase) in *Escherichia coli* K-12, J. Bacteriol. 162 (1985) 607–610.
[12] H. Asahara, P.M. Wistort, J.F. Bank, R.H. Bakerian, R.P. Cunningham, Purification and characterization of *Escherichia coli* endonuclease III from the cloned nth gene, Biochemistry 28 (1989) 4444–4449.
[13] D.M. Wilson III, B.P. Engelward, L. Samson, Prokaryotic base excision repair, in: J.A. Nickoloff, M.F. Hoekstra (Eds.), DNA Damage and Repair, vol. 1, Humana Press, Totowa, NJ, 1998, pp. 29–64.
[14] R. Aspinwall, D.G. Rothwell, T. Roldan-Arjona, C. Anselmino, C.J. Ward, J.P. Cheadle, J.R. Sampson, T. Lindahl, P.C. Harris, I.D. Hickson, Cloning and characterization of a functional human homolog of *Escherichia coli* endonuclease III, Proc. Natl. Acad. Sci. USA 94 (1997) 109–114.
[15] S. Ikeda, T. Biswas, R. Roy, T. Izumi, I. Boldogh, A. Kurosky, A.H. Sarker, S. Seki, S. Mitra, Purification and characterization of human NTH1, a homolog of *Escherichia coli* endonuclease III. Direct identification of Lys-212 as the active nucleophilic residue, J. Biol. Chem. 273 (1998) 21585–21593.
[16] T. Bessho, Nucleotide excision repair 3′ endonuclease XPG stimulates the activity of base excision repair enzyme thymine glycol DNA glycosylase, Nucleic Acids Res. 27 (1999) 979–983.
[17] A. Klungland, M. Hoss, D. Gunz, A. Constantinou, S.G. Clarkson, P.W. Doetsch, P.H. Bolton, R.D. Wood, T. Lindahl, Base excision repair of oxidative DNA damage activated by XPG protein, Mol. Cell 3 (1999) 33–42.
[18] A. Sancar, DNA excision repair, Annu. Rev. Biochem. 65 (1997) 43–81.
[19] W.L. de Laat, N.G. Jaspers, J.H. Hoeijmakers, Molecular mechanism of nucleotide excision repair, Genes Dev. 13 (1999) 768–785.
[20] A. O'Donovan, A.A. Davies, J.G. Moggs, S.C. West, R.D. Wood, XPG endonuclease makes the 3-prime incision in human DNA nucleotide excision repair, Nature 371 (1994) 432–435.
[21] T. Matsunaga, D. Mu, C.H. Park, J.T. Reardon, A. Sancar, Human DNA repair excision nuclease. Analysis of the roles of the subunits involved in dual incisions by using anti-XPG and anti-ERCC1 antibodies, J. Biol. Chem. 270 (1995) 20862–20869.
[22] D. Mu, D.S. Hsu, A. Sancar, Reaction mechanism of human DNA repair excision nuclease, J. Biol. Chem. 271 (1996) 8285–8294.
[23] D.R. Marenstein, M.T. Ocampo, M.K. Chan, A. Altamirano, A.K. Basu, R.J. Boorstein, R.P. Cunningham, G.W. Teebor, Stimulation of human endonuclease III by Y box-binding protein 1 (DNA-binding protein B). Interaction between a base excision repair enzyme and a transcription factor, J. Biol. Chem. 276 (2001) 21242–21249.
[24] D.R. Marenstein, M.K. Chan, A. Altamirano, A.K. Basu, R.J. Boorstein, R.P. Cunningham, G.W. Teebor, Substrate specificity of human endonuclease III (hNTH1). Effect of human APE1 on hNTH1 activity, J. Biol. Chem. 278 (2003) 9005–9012.
[25] M. Otterlei, E. Warbrick, T.A. Nagelhus, T. Haug, G. Slupphaug, M. Akbari, P.A. Aas, K. Steinsbekk, O. Bakke, H.E. Krokan, Post-replicative base excision repair in replication foci, EMBO J. 18 (1999) 3834–3844.
[26] A. Parker, Y. Gu, W. Mahoney, S.H. Lee, K.K. Singh, A.L. Lu, Human homolog of the MutY repair protein (hMYH) physically interacts with proteins involved in long patch DNA base excision repair, J. Biol. Chem. 276 (2000) 5547–5555.
[27] I. Boldogh, D. Milligan, M.S. Lee, H. Bassett, R.S. Lloyd, A.K. McCullough, hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs, Nucleic Acids Res. 29 (2001) 2802–2809.
[28] A.J. Giaccia, M.B. Kastan, The complexity of p53 modulation: emerging patterns from divergent signals, Genes Dev. 12 (1998) 2973–2983.
[29] L.J. Ko, C. Prives, p53: puzzle and paradigm, Genes Dev. 10 (1996) 1054–1072.
[30] C. Prives, Signaling to p53: breaking the MDM2-p53 circuit, Cell 95 (1998) 5–8.
[31] J. Zhou, J. Ahn, S.H. Wilson, C. Prives, A role for p53 in base excision repair, EMBO J. 20 (2001) 914–923.
[32] J.L. Manley, A. Fire, A. Cano, P.A. Sharp, M.L. Gefter, DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract, Proc. Natl. Acad. Sci. USA 77 (1980) 3855–3859.
[33] K. Fukuda, H. Morioka, S. Imajou, S. Ikeda, E. Ohtsuka, T. Tsurimoto, Structure–function relationship of the eukaryotic DNA replication factor, proliferating cell nuclear antigen, J. Biol. Chem. 270 (1995) 22527–22534.
[34] S. Benchimol, D. Pim, L. Crawford, Radioimmunoassay of the cellular protein p53 in mouse and human cell lines, EMBO J. 1 (1982) 1055–1062.
[35] S. Xie, Q. Wang, H. Wu, J. Cogswell, L. Lu, M. Jhanwar-Uniyal, W. Dai, Reactive oxygen species-induced phosphorylation of p53 on serine 20 is mediated in part by polo-like kinase-3, J. Biol. Chem. 276 (2001) 36194–36199.
[36] G. Maga, U. Hübscher, Proliferating cell nuclear antigen (PCNA): a dancer with many partners, J. Cell Sci. 116 (2003) 3051–3060.
[37] Y. Shimizu, S. Iwai, F. Hanaoka, K. Sugasawa, Xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase, EMBO J. 22 (2003) 164–173.
[38] C.E. Piersen, R. Prasad, S.H. Wilson, R.S. Lloyd, Evidence for an imino intermediate in the DNA polymerase beta deoxyribose

phosphate excision reaction, J. Biol. Chem. 271 (1996) 17811–17815.
[39] C.E. Piersen, A.K. McCullough, R.S. Lloyd, AP lyases and dRPases: commonality of mechanism, Mutat. Res. 459 (2000) 43–53.
[40] D.K. Srivastava, B.J. Berg, R. Prasad, J.T. Molina, W.A. Beard, A.E. Tomkinson, S.H. Wilson, Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps, J. Biol. Chem. 273 (1998) 21203–21209.
[41] G.L. Dianov, T. Thybo, I.I. Dianova, L.J. Lipinski, V.A. Bohr, Single nucleotide patch base excision repair is the major pathway for removal of thymine glycol from DNA in human cell extracts, J. Biol. Chem. 275 (2000) 11809–11813.
[42] L. Luna, M. Bjørås, E. Hoff, T. Rognes, E. Seeberg, Cell-cycle regulation, intracellular sorting and induced overexpression of the human NTH1 DNA glycosylase involved in removal of formamidopyrimidine residues from DNA, Mutat. Res. 460 (2000) 95–104.
[43] T. Haug, F. Skorpen, P.A. Aas, V. Malm, C. Skjelbred, H.E. Krokan, Regulation of expression of nuclear and mitochondrial forms of human uracil-DNA glycosylase, Nucleic Acids Res. 26 (1998) 1449–1457.