Production, Purification and Characterization of ^{15}N-Labeled DNA Repair Proteins as Internal Standards for Mass Spectrometric Measurements

Prasad T. Reddy*,[1], Pawel Jaruga*, Bryant C. Nelson[†], Mark S. Lowenthal*, Ann-Sofie Jemth[‡], Olga Loseva[‡], Erdem Coskun*, Thomas Helleday[‡], Miral Dizdaroglu*,[1]

*Biochemical Measurement Division and [†]Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA

[‡]Science for Life Laboratory, Division of Translational Medicine and Chemical Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

[1]Corresponding authors: e-mail address: prasad.reddy@nist.gov; miral@nist.gov

Contents

Abstract

Oxidatively induced DNA damage is caused in living organisms by a variety of damaging agents, resulting in the formation of a multiplicity of lesions, which are mutagenic and cytotoxic. Unless repaired by DNA repair mechanisms before DNA replication, DNA lesions can lead to genomic instability, which is one of the hallmarks of cancer. Oxidatively induced DNA damage is mainly repaired by base excision repair pathway with the involvement of a plethora of proteins. Cancer tissues develop greater DNA repair capacity than normal tissues by overexpressing DNA repair proteins. Increased DNA repair in tumors that removes DNA lesions generated by therapeutic agents before they became toxic is a major mechanism in the development of therapy resistance. Evidence suggests that DNA repair capacity may be a predictive biomarker of patient response. Thus, knowledge of DNA protein expressions in disease-free and cancerous tissues may help predict and guide development of treatments and yield the best therapeutic response. Our laboratory has developed methodologies that use mass spectrometry with isotope-dilution for the measurement of expression of DNA repair proteins in human and cultured cells. For this purpose, full length ^{15}N-labeled analogs of a number of human DNA repair proteins have been produced and purified to be used as internal standards for positive identification and accurate quantification. This article describes in detail the protocols of this work. The use of ^{15}N-labeled proteins as internal standards for the measurement of several DNA repair proteins *in vivo* is also presented.

1. INTRODUCTION

Exogenous and endogenous sources such as free-radicals and ionizing radiation generate oxidatively induced DNA damage by a variety of mechanisms, resulting in the formation of modified bases and sugars, DNA-protein cross-links, strand breaks, base-free sites, and tandem lesions such as 8,5'-cyclopurine-2'-deoxyribonucleosides and clustered damaged sites (reviewed in (Dizdaroglu & Jaruga, 2012, Evans *et al.*, 2004)). This type of DNA damage is thought to play an important role in disease processes such as carcinogenesis and aging (reviewed in (Dizdaroglu, 2015, Friedberg *et al.*, 2006)). Elaborate DNA repair pathways exist in mammalian cells with approximately 150 different proteins involved (reviewed in (Friedberg *et al.*, 2006, Wood *et al.*, 2005)). DNA repair deficiencies cause accumulation of DNA damage and mutations, leading to genomic instability, which is a major factor in carcinogenesis (Beckman & Loeb, 2005, Helleday *et al.*, 2008, Hoeijmakers, 2001, Kelley, 2012, Liu *et al.*, 2007, Loeb, 2011, Madhusudan & Middleton, 2005). Oxidatively induced DNA damage is mainly repaired by base excision repair (BER) and also by nucleotide excision repair (NER), albeit to a lesser extent (Friedberg, Walker, Siede, Wood, Schultz, & Ellenberger, 2006). In the first step of BER, DNA glycosylases hydrolyze the N-glycosidic bond releasing the damaged base and generating an abasic site, followed by the action of a series of other BER enzymes (Gros *et al.*, 2002, Hegde *et al.*, 2008). Formamidopyrimidine DNA glycosylase (Fpg, also called MutM) is one of the main DNA glycosylases in *Escherichia coli*, which specifically excises 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-hydroxyguanine (8-OH-Gua) from DNA containing multiple lesions (Boiteux *et al.*, 1992, Dizdaroglu, 2005). In eukaryotes, 8-oxoguanine-DNA glycosylase (OGG1) including human OGG1 (hOGG1), which is a functional homolog of Fpg, exhibits a strong specificity for FapyGua and 8-OH-Gua, but does not act on FapyAde (Audebert *et al.*, 2000, Dherin *et al.*, 1999, Hu *et al.*, 2005, Sidorenko *et al.*, 2009).

Mammalian NEIL1 protein, which is a *E. coli* Nei-like DNA glycosylase with an additional β,δ-elimination activity, has been discovered in eukaryotes (Bandaru *et al.*, 2002, Hazra *et al.*, 2002). This enzyme is unique in that it specifically removes FapyAde and FapyGua from DNA with multiple lesions, without exhibiting any significant activity for 8-OH-Gua (Bandaru *et al.*, 2002, Chan *et al.*, 2009, Doublie *et al.*, 2004, Hazra *et al* ., 2002, Hu *et al.*, 2005, Jaruga *et al.*, 2004, Liu *et al.*, 2010, Muftuoglu *et al.*, 2009, Rosenquist *et al.*, 2003, Roy *et al.*, 2007). The

involvement of NEIL1 in NER has also been suggested on the basis of accumulation of 8,5'-cyclopurine-2'-deoxyribonucleosides in *neil1*$^{-/-}$ mice (Jaruga *et al.*, 2010). Homologues of *E. coli* Nth have also been found in yeast and mammals (Aspinwall *et al.*, 1997, Roldán-Arjona *et al.*, 1996). Mammalian NTH1 mainly acted on pyrimidine-derived lesions, exhibiting a narrower substrate specificity than *E. coli* Nth (Karahalil *et al.*, 1998, Roldán-Arjona *et al.*, 1996); however, evidence has also been provided for purine-derived FapyAde to be the physiological substrate of NTH1 (Chan *et al.*, 2009, Hu *et al.*, 2005).

Other enzymes such as apurinic/apyrimidinic endonuclease 1 (APE1) and DNA polymerase β (Pol β) also plays vital roles in BER. Abasic sites, which are left behind following removal of modified DNA bases by DNA glycosylases, are processed by apurinic/apyrimidinic endonuclease 1 (APE1) (Demple *et al.*, 1991). APE1 provides over 95 % of the total apyrimidinic/apurinic endonuclease function with some additional critical functions (Abbotts & Madhusudan, 2010, Barnes *et al.*, 2009, Demple *et al.*, 1991, Demple & Harrison, 1994, Fishel *et al.*, 2013, Gros *et al.*, 2004, Tell *et al.*, 2009, Wilson, III & Barsky, 2001, Xanthoudakis & Curran, 1992). The action of APE1 generates a one-nucleotide gap with a 3'-OH and a 5'-terminal 2'-deoxyribose phosphate (dRP) moiety. Pol β, which is found in all vertebrate species (Beard & Wilson, 2006, Beard & Wilson, 2014, Braithwaite & Ito, 1993), binds to this gap, and performs DNA synthesis with its DNA polymerase activity, filling in the gap. It then removes the blocking dRP-moiety, paving the way for other enzymes such as DNA ligases to close the resulting gap to complete the repair of DNA (Beard & Wilson, 2000, Beard & Wilson, 2006, Matsumoto & Kim, 1995, Srivastava *et al.*, 1998).

Defects in BER enzymes such as those discussed above are associated with neurological disorders and cancer (Sampath *et al.*, 2012, Wallace *et al.*, 2012, Wilson, III *et al.*, 2011). Polymorphic variants of these enzymes have been found in human populations in connection to various cancer incidences (reviewed in (Dizdaroglu, 2015)). For example, *neil1*$^{-/-}$ mice developed obesity and symptoms of metabolic syndrome (Vartanian *et al.*, 2006). Male and female *neil1*$^{-/-}$ and *nth1*$^{-/-}$ mice developed pulmonary and hepatocellular tumors to an extent up to ≈ 15 %. A dramatic increase up to 75 % in cancer incidence has been observed in *neil1*$^{-/-}$ and *nth1*$^{-/-}$ male and female mice (Chan *et al.*, 2009). In mice with both deleted alleles of *ape1*, early embryonic lethality has been observed; similarly, APE1 heterozygous mice exhibited increased oxidative stress, spontaneous mutagenesis and cancer incidences, and reduced survival of pups

and embryos (Cabelof, 2012, Hakem, 2008, Huamani *et al.*, 2004, Meira *et al.*, 2001, Xanthoudakis *et al.*, 1996). Thus, accumulated evidence unequivocally points to the crucial role of BER enzymes in the maintenance of genetic stability and disease prevention.

It is now well known that overexpression of DNA repair proteins that may increase the DNA repair capacity is common in cancer (Helleday *et al.*, 2008, Kelley, 2012, Madhusudan & Middleton, 2005). Increased and effective DNA repair capacity in tumors that removes DNA lesions generated by ionizing radiation and/or chemotherapy before they became toxic is a major mechanism to develop therapy resistance. Evidence suggests that DNA repair capacity might be a predictive biomarker of patient response (Kelley, 2012, Perry *et al.*, 2012). In this respect, BER proteins have emerged as biomarkers for assessment of the effectiveness and outcome of therapy (Chan & Bristow, 2010, Curtin, 2013, Fishel, Vascotto, & Kelley, 2013, Helleday, 2008, Helleday *et al.*, 2008, Illuzzi & Wilson, III, 2012, Kelley, 2012, Madhusudan & Middleton, 2005, Moeller *et al.*, 2010, Perry *et al.*, 2012). Targeting MTH1 protein, which is not a BER protein, has been also suggested to be beneficial in cancer therapy (Gad *et al.*, 2014, Huber *et al.*, 2014). This enzyme dephosporylates modified 2'-deoxynucleoside triphosphates in the nucleotide pool so that they cannot be incorporated into DNA during DNA replication, preventing cell death and mutations (Maki & Sekiguchi, 1992, Mo *et al.*, 1992, Sakai *et al.*, 2002, Sakumi *et al.*, 1993, Sekiguchi & Tsuzuki, 2002, Tsuzuki *et al.*, 2001). Its overexpression has been observed in many cancers (Gad *et al.*, 2014, Huber *et al.*, 2014, Kennedy *et al.*, 1998, Okamoto *et al.*, 1996, Speina *et al.*, 2005).

The determination of the overexpression or underexpression of DNA repair proteins in normal and cancer tissues may help predict and guide development of treatments, potentially yielding the greatest therapeutic response (Kelley, 2012). In general, expression levels of DNA repair proteins have been estimated by Western blot methods. With these methods, proteins are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by the use of an antibody specific to a target protein, meaning that they completely depend on a reliable and specific antibody resource. Antibodies may potentially exhibit some off-target binding, leading to false results. No positive identification and no absolute quantification are provided because of the lack of mass spectrometric evidence and suitable internal standards. Quantification is based on the estimation of stained area only. The molecular mass of a target protein is only estimated by the use of a molecular mass ladder of proteins. If

DNA repair proteins are to be used as reliable biomarkers in cancer, their expression levels must be accurately measured in tissues, providing positive identification of a target protein and its absolute quantification. Mass spectrometry is the most suitable technique of choice for the measurement of proteins. The application of this technique would be essential for positive identification and accurate quantification of DNA repair proteins in human tissues using proper internal standards. Our laboratory has recently developed methodologies and stable isotope-labeled standards for the measurements of DNA repair proteins using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with isotope-dilution (Coskun *et al.*, 2015, Dizdaroglu *et al.*, 2011, Kirkali *et al.*, 2013, Reddy *et al.*, 2011, Reddy *et al.*, 2013). Stable isotope-labeled analogs of DNA repair proteins, which are to be used as internal standards, were produced, purified and characterized. This article describes the procedures used for this purpose and the application of the developed internal standards for the measurement of DNA repair proteins.

2. MATERIALS AND METHODS

2.1. Materials

Restriction endonucleases, DNA ligase, and calf intestinal alkaline phosphatase were obtained from New England Biolabs (MA). Protease inhibitors were the product of Roche. Isopropyl β-D-thiogalactoside (IPTG), acrylamide, bisacrylamide and Tris (2-carboxyethyl)phosphine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). Diethylaminoethyl (DEAE) cellulose (DE52) was from Whatman laboratories. Shodex carboxymethyl cellulose HPLC preparative column (CM 2025) was from Phenomenex. YM10 membrane filters were from EMD Millipore Corporation (Bedford, MA). Ni-Sepharose column HisTrap HP and MonoQ anion-exchange column were obtained from GE Healthcare, Bio-Science (Uppsala, Sweden). BugBuster protein extraction reagent was from Millipore (Billerica, MA). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). Benzonase nuclease was from Novagen (Denmark).

2.2. *Escherichia coli* strains and plasmids

The pET11a expression vector, for native protein production of *Ec*Fpg based on the IPTG induction, was obtained from Navagen, Madison, WI. The relevant strains for cloning and expression are *E. coli* Novablue (K12) and BL21(DE3), respectively. The human *ogg1* gene in

the expression vector pET15b for his-tagged production of the protein was kindly provided by Dr. Dmitry Zharkov through Prof. Arthur Grollman's laboratory. The human *neil1* gene in the expression vector pET28 for His-tagged production of the protein was kindly provided by Dr. Stephen Lloyd (Oregon Health and Science University, Portland, Oregon). The human *ape1* gene was provided by Dr. David M. Wilson III of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA.

2.3. Preparation of minimal medium

The composition of the medium was: 6 g NaH_2PO_4, 3 g K_2HPO_4, 0.5 g NaCl, and 1 g ^{15}N-NH_4Cl, 5 g glucose, 246 mg $MgSO_4.7H_2O$ per liter. Ampicillin or kanamycin was added to a final concentration of 50 µg/mL or 25 µg/mL, respectively.

2.4. DNA procedures

E. coli Novablue (K12) harboring a recombinant plasmid was grown at 37 ^{o}C overnight in 10 mL Luria-Bertani medium (Miller, 1972), containing the appropriate antibiotic. Minipreparations of plasmid DNA were purified using a Qiagen Kit. Digestion of DNA with restriction enzymes was performed according to the manufacturer's recommendation. DNA fragments were separated by electrophoresis on SeaKem GTG agarose or NuSieve GTG agarose. DNA was purified using a Qiagen Kit. Ligation of DNA fragments was performed as described (Sambrook *et al.,* 1989). Competent cells of the *E. coli* strains used here were prepared by the Hanahan method (Hanahan, 1983, Sambrook *et al.,* 1989).

3. PRODUCTION AND PRUFICATION OF *E. COLI* ^{15}N-FPG PROTEIN

3.1. Cloning of *fpg* into pET11a vector

The coding sequence for *E. coli* Fpg gene from the plasmid pFpg was provided by Dr. Timothy R. O'Connor of the Beckman Research Institue of the City of Hope, Duarte, CA (O'Connor *et al.,* 1989). It was amplified by PCR with Pfu polymerase using a 5' end primer (5'-GGAATTC CAT ATG CCT GAA TTA CCC GAA G-3') and a 3' end primer (5'-CCG CTC GAG TTA CTT CTG GCA CTG CCG AC-3'). The 5' end primer sequence contained the NdeI restriction recognition sequence CATATG wherein ATG served as the initiation codon for protein expression. The 3' end primer contained the TAA translation stop codon. The amplified product was digested with NdeI to produce

the gene with 5' NdeI protruding end and 3' blunt end. The DNA fragment was purified from a 1 % agarose gel, and cloned into the NdeI and BamHI (first digested with BamHI, filled in with DAN polymerase to make this blunt end, then digested with NdeI) sites of the pET11a expression vector. A recombinant plasmid pET11a/Fpg was isolated from *E. coli* Nova Blue cells. *E.coli* BL21(DE3) was transformed with the recombinant plasmid to induce protein expression with IPTG.

3.2. Production of ^{15}N-Fpg

E. coli BL21(DE3) harboring pET11a/Fpg recombinant plasmid was grown at 37 ºC for 20 h on LB agar plate containing 100 µg/mL ampicillin. A colony was carefully (without touching into the LB medium) transferred to 10 mL minimal medium containing 1 mg ^{15}N-NH_4Cl/mL and 50 µg ampicillin/mL. Cells were grown for 1 h at 37 ºC at 250 rpm in a 50 mL tube. This inoculum was transferred to 140 mL minimal medium, in a 500 mL baffled flask, containing ^{15}N-NH_4Cl and ampicillin as above. This culture was grown at 37 ºC for 16 h. Next, 3 x 50 mL of this seed culture was transferred to 3 x 450 mL of minimal medium containing ^{15}N-NH_4Cl and ampicillin in 3 x 2 L baffled flasks. IPTG was added to a final concentration of 30 µmol/L to induce Fpg production at 37 ºC for 1 h. Next, the culture was shifted to 42 ºC for 5 h to continue ^{15}N-Fpg production. Cells were harvested at 6000 x g for 20 min and washed with 25 mmol/L Tris-Cl buffer, pH, 7.5. The wet weight of cells obtained in this procedure was ≈3 g.

3.3. Purification of ^{15}N-Fpg

Cell pellet was suspended in 40 ml of 50 mmol/L Tris-Cl pH 8.0, 10 mmol/L β-mercaptoethanol, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 50 mmol/L NaCl, (lysis buffer 1) containing one tablet of protease inhibitors. Cell suspension was passed through a French Press at 7.1 x 10^4 kPa twice. The cell-free extract was centrifuged at 10000 x g for 20 min. The supernatant was mixed with 5 g of DE52 anion exchange resin equilibrated with the lysis buffer 1 in a 500 mL bottle at 80 rpm for 1 h at 4 ºC and then poured into a column. The flow through containing nearly all the Fpg and fewer cellular proteins was collected. The resin was washed with 10 mL of the lysis buffer 1 and added to the flow through. The Fpg enriched pool (50 mL) was dialyzed overnight against 1 L of 20 mmol/L potassium phosphate buffer, pH 7.4. The dialyzed pool was centrifuged at 100000 x g for 1 h to remove any particulate material. The supernatant was chromatographed on a HPLC-Shodex caboxymethyl cellulose column (2 cm x

20 cm) equilibrated with 20 mmol/L potassium phosphate buffer, pH 7.4. The column was washed with 100 mL of 20 mmol/L potassium phosphate buffer, pH 7.4 until the A_{280} stabilized. Then Fpg was eluted with a 0 to 0.5 mol/L potassium chloride gradient (250 mL each) in 20 mmol/L potassium phosphate buffer, pH 7.4. Pure Fpg was eluted as a sharp peak at ≈ 0.3 mol/L KCl. Fractions containing Fpg were pooled, concentrated on YM3 membrane filter, and dialyzed overnight against 500 mL of 50 % glycerol, 20 mmol/L Tris-Cl, 1 mmol/L EDTA, and 10 mmol/L β-mercaptoethanol, pH 7.5. Protein concentration was determined by the Lowry method using BSA as standard (Lowry *et al.*, 1951). Total yield of the protein was 1 mg. The SDS-PAGE and Coomassie staining of ^{15}N-Fpg (lane 5) and protein molecular mass standards (lane 6) is shown in Figure 1, indicating high purity of the labeled protein (Reddy *et al.*, 2011).

4. PRODUCTION AND PURIFICATION OF ^{15}N-hOGG1

4.1. Production of ^{15}N-hOGG1

E. coli BL21(DE3) harboring pET15b/hOGG1 recombinant plasmid was grown at 37 ºC for 20 h on LB agar plate containing 100 µg/mL ampicillin. A colony was carefully (without touching into the LB medium) transferred to 100 mL minimal medium containing 1 mg ^{15}N-NH_4Cl/mL and 50 µg ampicillin/mL. Cells were grown overnight (16 h) at 37 ºC at 250 rpm in a 250 mL baffled flask. This inoculum was transferred to 1000 mL minimal medium, in a 2000 mL baffled flask, containing ^{15}N-NH_4Cl and ampicillin as above. This culture was grown at 37 ºC for 1 h and briefly cooled in ice water to room temperature. Next, IPTG was added to a final concentration of 100 µmol/L to induce hOGG1 production at 24 ºC overnight. Cells were harvested at 6000 x g for 20 min and washed with 25 mmol/L Tris-Cl buffer, pH: 7.5. The wet weight of cells obtained in this procedure was ≈2 g.

4.2. Purification of ^{15}N-hOGG1

The cell pellet was suspended in 20 mL of 50 mmol/L Tris-Cl, pH: 8.0, 10 mmol/L β-mercaptoethanol, 300 mmol/L NaCl, and 10 mmol/L imidazole (lysis buffer 2) containing one tablet of protease inhibitors. Cell suspension was passed through a French Press at 7.1 x10^4 kPa. The cell-free extract was centrifuged at 40000 x g for 1 h. Meanwhile, 2 mL of nickel-agarose slurry (1mL resin) was washed with the lysis buffer 2 in a 30 mL Bio-Rad polypropylene column. The supernatant was added to the resin and mixed on a rocker for 2 h at 4 ºC. The flow

through was collected, and the column was washed successively with three 10 mL aliquots of the lysis buffer 2. Next, the resin was washed three times with 3 mL aliquots of the lysis buffer 2 containing an additional 20 mmol/L imidazole (total 30 mmol/L). Next, the hOGG1 was eluted with five 5 mL aliquots of the lysis buffer containing 100 mmol/L imidazole. The first 5 mL contained about 50 % of hOGG1 (≈95 % pure) with minor contaminants as judged by SDS-PAGE. The subsequent four elutions contained the remaining hOGG1 with higher purity (≈98 %). All the five elutions containing hOGG1 were pooled, concentrated on YM3 membrane filter, and dialyzed overnight against 500 mL of 50 % glycerol, 20 mmol/L Tris-Cl, 1 mmol/L EDTA, and 10 mmol/L β-mercaptoethanol, pH 7.5. Mass measurements and activity analysis of ^{15}N-hOGG1 was carried out. Protein concentration was determined by the Lowry method using BSA as standard (Lowry *et al*., 1951). Total yield of the protein was 5.3 mg. Figure 2 shows the SDS-PAGE and Coomassie staining of ^{15}N-hOGG1 (lane 5) and protein molecular mass standards (lane 6), indicating high purity of the labeled protein. (Reddy *et al.*, 2011).

5. PRODUCTION AND PURIFICATION OF ^{15}N-hNEIL1

5.1. Production of ^{15}N-hNEIL1

E. coli BL21 (DE3) harboring pET28 / human *neil1* recombinant plasmid was grown at 37 °C for 20 h on LB agar plate containing 100 µg ampicillin and 40 µg chloramphenicol /mL. A colony was carefully (without touching into the LB medium) transferred to 10 mL minimal medium containing 1 mg ^{15}N-NH_4Cl/mL and 50 µg ampicillin and 20 µg chloramphenicol/mL. Cells were grown for 16 h at 37 °C at 250 rpm in a 50 mL tube. This inoculum was transferred to 90 mL minimal medium, in a 250 mL flask, containing ^{15}N-NH_4Cl, ampicillin, and chloramphenicol as above. This culture was grown at 37 °C for 9 h. Next, this seed culture was transferred to 900 mL of minimal medium containing ^{15}N-NH_4Cl, ampicillin, chloramphenicol in a 2 L flask. This culture was grown at 37 °C for 4 h. Cells (A_{600} = 0.45) were briefly cooled in ice and ^{15}N-hNEIL1 production was induced with 100 µmol IPTG/mL at 25 °C for 16 h. Cells were harvested at 6000 x g for 20 min and washed with 25 mmol/L Tris buffer (pH 7.5). The wet weight of cells obtained in this procedure was 2.8 g/L culture.

5.2. Purification of ^{15}N-hNEIL1

Cell pellet was suspended at a rate of 1 g/10 mL of 50 mmol/L Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0), 10 mmol/L β-mercaptoethanol, 10 mmol/L imidazole, and 300 mmol/L NaCl (lysis buffer). One tablet of protease inhibitors was added to the cell suspension. Cell suspension was passed through a French Press at 7.1 $x10^4$ kPa twice. The cell-free extract was centrifuged at 36000 x g for 30 min. Meanwhile, 1 mL of nickel-agarose slurry (0.5 mL resin) was washed with the lysis buffer in a 30 mL Bio-Rad polypropylene column. The supernatant was added to the resin and mixed on a rocker for 2 h at 4 °C. The flow through was collected, and the column was washed successively with three 10 mL aliquots of the lysis buffer. Next, the resin was washed successively two times with 10 mL aliquots of the lysis buffer containing an additional 30 mmol/L imidazole (total 40 mmol/L). Next, the resin was washed successively three times with 5 mL aliquots of the lysis buffer containing an additional 70 mmol/L imidazole (total 80 mmol/L). The first $imidazole_{80}$ wash did elute about 30 % of ^{15}N-hNEIL1 with a number of contaminating proteins as judged by SDS-PAGE. The second and third $imidazole_{80}$ washes eluted about 30 % of nearly homogeneous ^{15}N-hNEIL1. Next, ^{15}N-hNEIL1 was eluted successively with three 5 mL aliquots of the lysis buffer containing 150 mmol/L imidazole. The first 5 mL contained about 30 % of ^{15}N-hNEIL1. The subsequent two elutions contained the remaining ^{15}N-hNEIL1. The second and third $imidazole_{80}$ elutions and all the $imidazole_{150}$ elutions were pooled and dialyzed overnight against 1 L of 50 mmol/L Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0) and 300 mmol/L NaCl. Slight precipitate of ^{15}N-hNEIL1 appeared after dialysis even in the presence of 300 mmol/L NaCl. The precipitate was centrifuged off from the dialyzed pool and the clear supernatant was concentrated on NMLW5 membrane in an Amicon cell. The above purification procedure developed for cells from 1 L culture was proportionately scaled up to 5 L as and when needed. Protein concentration was determined by the Lowry method using BSA as the standard (Lowry *et al.*, 1951). The final yields of ^{15}N-hNEIL1 was ≈2 mg/L culture. The SDS-PAGE and Coomassie staining of ^{15}N-hNEIL1 (lane 5) and protein molecular mass standards (lane 6), is presented in Figure 3. A high purity of the labeled protein can be seen with a minor contaminant at ≈17 kDa (Reddy *et al.*, 2013).

6. PRODUCTION AND PURIFICATION OF ^{15}N-hAPE1

6.1. Production of ^{15}N-hAPE1

E. coli BL21 (DE3) harboring pET11/h*APE1* recombinant plasmid was grown at 37 °C for 20 h on LB agar plate containing 100 µg ampicillin/mL. A colony was carefully (without touching into the LB medium) transferred to 10 mL minimal medium containing 50 µg ampicillin/mL. Cells were grown for 3 h at 37 °C at 250 rpm in a 50 mL tube. This inoculum was transferred to 200 mL minimal medium with 50 µg ampicillin/mL in a 500 mL flask. This culture was grown at 37 °C for 14 h. Next, each 50 mL of this seed culture was transferred to 4 x 1000 mL of minimal medium containing ^{15}N-NH_4Cl and ampicillin in 4 x 2 L flasks. This culture was grown at 37 °C for 6 h and then at 20 °C for 90 min. Cell density at this stage was A_{600} = 0.5. The production of ^{15}N-hAPE1 was induced with 100 µmol IPTG / mL at 20 °C for 15 h. Cells were harvested at 6000 x g for 20 min and washed with 25 mmol/L Tris buffer (pH 7.5). The wet weight of cells obtained in this procedure was 2.25 g/L culture for a total of 9 g wet weight of cells.

6.2. Purification of ^{15}N-hAPE1

Nine grams of cells from 4 L culture were suspended in 90 mL of lysis bufferA: 50 mmol/L HEPES-KOH buffer, pH: 7.5, containing 5 % glycerol, 50 mmol/L KCl, and 1 mmol/L DTT. Cells were broken by passing through a French Press at 7.1 x 10^4 kPa. The cell-free extract was centrifuged at 70000 x g for 1 h. The supernatant was mixed with 5 g of DE52 anion exchange resin equilibrated with the buffer A. The supernatant / resin slurry in a 250 ml bottle was mixed at 178 rpm for 1 h and then poured into a column. The flow through containing nearly all the hAPE1 and fewer cellular proteins was collected. The resin was washed with 10 mL of the buffer A and added to the flow through. The ^{15}N-hAPE1 enriched pool (100 mL) was chromatographed on a HPLC-Shodex caboxymethyl cellulose column (2.0 cm x 25 cm) equilibrated with Buffer A. The column was washed with 100 mL of Buffer A until the A_{280} stabilized. Then ^{15}N-hAPE1 was eluted with a potassium chloride gradient generated from Buffer A and Buffer A containing 0.65 mol/L KCl (250 mL each). Pure ^{15}N-hAPE1 was eluted as a sharp peak at ≈ 0.3 mol/L KCl. Fractions containing ^{15}N-hAPE1 were pooled, concentrated to ≈10 mg/mL on a YM10 membrane filter. The yield of ^{15}N-hAPE1 from 4 L culture of minimal medium was 60 mg. Aliquots of the protein was stored at -70 ^{o}C. Figure 4, lane 6, shows the SDS-PAGE analysis and

Coomassie staining of ^{15}N-hAPE1 and protein molecular mass standards (lane 7), indicating high purity of the labeled protein (Kirkali *et al.*, 2013).

7. PRODUCTION AND PURIFICATION OF ^{15}N-hMTH1

^{15}N-hMTH1 was produced according to the procedures used for the production of hMTH1 (Svensson *et al.*, 2011). ^{15}N-hMTH1 was expressed from pET28a-hMTH1 in *E.coli* BL21(DE3) grown in a minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 5 g glucose, 120.4 mg $MgSO_4$ per liter) containing 1 g ^{15}N-NH_4Cl. The production was induced by addition of 200 µmol/L isopropyl β-D-1-thiogalactopyranoside (Sigma, St. Louis, MO). Cells were harvested by centrifugation after 16 h at 20 °C and the obtained pellet was dissolved in BugBuster protein extraction reagent supplemented with complete protease inhibitor cocktail and benzonase nuclease. The resulting suspension was centrifuged and the cleared lysate was subjected to chromatography on Ni-Sepharose column HisTrap HP. Bound proteins were eluted using a linear gradient of imidazole (25 mmol/L – 500 mmol/L) and analyzed by SDS-PAGE. Fractions containing ^{15}N-hMTH1 were after dialysis loaded onto a MonoQ anion-exchange column and eluted with a linear gradient of NaCl. Eluted fractions were analyzed using SDS-PAGE with Coomassie staining. Fractions containing ^{15}N-hMTH1 protein were pooled and stored in 20 mmol/L HEPES, 225 mmol/L NaCl, 10 % glycerol and 1 mmol/L Tris (2-carboxyethyl)-phosphine hydrochloride at –80 °C. The purity of the isolated ^{15}N-hMTH1 was checked using SDS-PAGE with Coomassie staining. Figure 5 illustrates the SDS-PAGE analysis of ^{15}N-hMTH1 along with hMTH1 and protein standards, indicating high purity of both proteins (Coskun *et al.*, 2015).

8. MASS SPECTROMETRIC ANALYSIS OF ^{15}N-LABELED PROTEINS

Mass spectrometry was used to measure the molecular masses and to check the isotopic purities of ^{15}N-labeled DNA repair proteins that were produced as described above. MALDI-ToF mass spectrometry was applied to measure the molecular masses of *E. coli* ^{15}N-Fpg and ^{15}N-hOGG1 (Reddy *et al.*, 2011). The protonated average molecular mass (MH^+) of *E. coli* ^{15}N-Fpg amounts to 30641 Da, as calculated using the *NIST Mass and Fragment Calculator*. The measurement yielded a value of 30442 Da. It is well known that the N-terminal Met is removed from proteins by peptide deformylase and methionineaminopeptidase as an essential process,

when the penultimate residue is small and uncharged as Pro next to Met (Giglione *et al.*, 2004). Without the N-terminal Met, the calculated value of the MH^+ of *E. coli* ^{15}N-Fpg amounted to 30509 Da. The measured value (30442 Da) is 99.8 % of this calculated value. Using the same approach, the MH^+ of His-tagged ^{15}N-hOGG1 was found to be 41834 Da. The His-tag had a sequence of MGSSHHHHHHSSGLVPRGSHEL. The calculated MH^+ of this protein amounts to 41688 Da, which is 99.7 % of the measured value. The measured MH^+ of His (KLAAALEHHHHHH)-tagged ^{15}N-hNEIL1 amounted to 45833 Da (unpublished results). The calculated MH^+ of this protein was 45785 Da, which is 99.9 % of the measured value. Considering that the MALDI-ToF mass spectrometry provides a mass accuracy of 0.1 %, these values represent more than 99 % ^{15}N-labeling of *E. coli* Fpg, hOGG1 and hNEIL1 proteins.

An Orbitrap mass spectrometer coupled to an HPLC system, which has a mass accuracy of 0.001 %, was used to measure the molecular mass of ^{15}N-hAPE1 (Kirkali *et al.*, 2013). The calculated MH^+ of ^{15}N-hAPE1 with the loss of Met amounts to 35858 Da. The measured MH^+ was 35854 Da, which differs by 4 Da only from the calculated value and thus represents a 99.99 labeling. Within the mass accuracy of the Orbitrap mass spectrometer, this result showed that a complete ^{15}N-labeling of hAPE1 was achieved.

The molecular mass of ^{15}N-hMTH1 was measured using a high resolution QToF mass spectrometer coupled to an HPLC system (Coskun *et al.*, 2015). Without the N-terminal Met, the calculated MH^+ of ^{15}N-hMTH1 amounts to 20230.9 Da. The measured MH^+ of ^{15}N-hMTH1 was 20216.2 Da, which is 99.93 % of the calculated value. Taken together, the measurement of the average molecular masses of the produced full length ^{15}N-labeled proteins confirmed their identity and an almost complete ^{15}N-labeleing, meaning that these labeled protein can be used as excellent internal standards for the measurement of DNA repair proteins by LC-MS/MS or any other MS technique.

9. APPLICATIONS TO THE MEASUREMENT OF DNA REPAIR PROTEINS

9.1. Development of methodologies

Following production of ^{15}N-labeled DNA repair proteins, we developed methodologies using these proteins as internal standards to positively identify and accurately quantify corresponding DNA repair proteins in human cultured cells and tissues with the use of LC-MS/MS. For this purpose, ^{15}N-labeled proteins and their unlabeled analogs were hydrolyzed with

trypsin and then analyzed by LC-MS/MS. A number of tryptic peptides of each protein were identified on the basis of their full scan mass spectra and product ion spectra. Identified tryptic peptides yielded protein scores of more than 100 with the use of the "Mascot" search engine (http://www.matrixscience.com), according to which protein scores greater than 70 are considered significant ($p < 0.05$) for positive identification. Moreover, using to the SwissProt database (http://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi) and the taxonomy *Homo sapiens* (20200 sequences), several combinations of only four out of many identified tryptic peptides provided a 100% match with studied proteins. This means that the simultaneous measurement of just four tryptic peptides would be sufficient to positively identify and quantify a target protein. As an example of full scan mass spectra, Figure 6 illustrates the mass spectrum of one of the tryptic peptides of hAPE1 and its ^{15}N-labeled analog (Kirkali *et al.*, 2013). In general, mass spectra of such peptides contain an MH^+ ion of lower abundance (ion at *m/z* 1131 in Figure 6) and a doubly protonated (charged) molecular ion [$(M+2H)^{2+}$] as the most abundant ion (*m/z* 566.1 in Figure 6) (Kinter & Sherman, 2000). The mass spectrum of the ^{15}N-labeled peptide exhibited a shift of 14 Da in the mass of MH^+ of the unlabeled peptide, consistent with the fourteen ^{15}N atoms in the molecule. The shift in the mass of $(M+2H)^{2+}$ was also in agreement with the mass difference of 14 Da. To obtain product ion spectra, optimum collision energies using $(M+2H)^{2+}$ ions as the precursor ions were measured (Coskun *et al.*, 2015, Reddy *et al.*, 2013). These were in agreement with previously determined collision energies of peptides with similar masses (Kinter & Sherman, 2000). Product ion spectra of identified tryptic peptides were recorded using the determined collision energy for each peptide. As examples, product ion spectra of the peptides in Figure 6 are illustrated in Figure 7 (Kirkali *et al.*, 2013). Such spectra are generally dominated by so-called *y*-series ions with *b*-series ions being of lesser intensity (Kinter & Sherman, 2000). In Figure 7A, typical *y*-ions from the y_1-ion to the y_9-ion, with the most intense ion being the y_8-ion at *m/z* 904 are seen with only a few *b*-ions. The ^{15}N-labeled analog gave an essentially identical spectrum with mass shifts according to the ^{15}N-content of the fragments (Figure 7B). The observed masses of all *y*-series ions and *b*-series ions in product ion spectra of analyzed tryptic peptides perfectly matched the corresponding theoretical masses calculated using *NIST Mass and Fragment Calculator* (www.nist.gov/mml/bmd/bioanalytical/massfragcalc.cfm).

Selected-reaction monitoring (SRM) was used to analyze trypsin hydrolysates of proteins and their ^{15}N-labeled analogs to establish the sensitive and accurate measurement of proteins *in vivo*. Mass

transitions from $(M+2H)^{2+}$ to the most intense *y*-ion in product ion spectra were used. Ion-current profiles of the mass transitions of tryptic peptides exhibited excellent peak shapes and a base-line separation between peptides. As expected, each tryptic peptide and its respective ^{15}N-labeled analogue co-eluted at the same retention time (Dizdaroglu *et al.*, 2011, Reddy *et al*., 2013, Coskun *et al.*, 2015, Kirkali *et al.*, 2013). To further ascertain the identity of a target peptide, multiple typical transitions were simultaneously monitored. All typical transitions of tryptic peptides lined up at their appropriate retention times, confirming their identity. The analytical sensitivity of the LC-MS/MS instrument was measured. Depending on the peptide, the on-column limit of detection varied from 10 fmol to 20 fmol with a signal-to-noise ratio of at least 3, whereas the limit of quantification was approximately 50 fmol to 100 fmol of target peptide with a signal-to-noise ratio of 10.

9.2. Measurement of DNA repair proteins *in vivo*

hAPE1 has been positively identified and quantified in nuclear and cytoplasmic extracts of three human cell lines and in mouse liver (Kirkali *et al*., 2013), and in human cancerous and disease-free breast tissues (unpublished data). Different expression levels of hAPE1 were observed depending of the cell line and tissue types. As an example, Figure 8 illustrates ion-current profiles of mass transitions of five tryptic peptides of hAPE1 and ^{15}N-hAPE1 obtained using the tryptic digest of a nuclear extract of human mammary gland epithelial adenocarcinoma (MCF-7) cells. The signals of five peptides of hAPE1 and ^{15}N-hAPE1, which was added to protein extracts as an internal standard, lined up at the corresponding retention times, unequivocally identifying hAPE1 in this human cell line. The expression levels of hAPE1 in cell lines and tissues were calculated by using the measured signals of mass transitions of each tryptic peptide and their ^{15}N-labeled analogs, the amount of the internal standard, and the protein amount in each protein extract. In addition, we demonstrated the detection of APE1 variants with single amino acid replacements, i.e., Gln51His and Gly241Arg, which occur in several cancers (Pieretti *et al.*, 2001), and in the general population (Hadi *et al.*, 2000, Mohrenweiser *et al.*, 2002).

hMTH1 has been unequivocally identified and quantified in human malignant and disease-free breast tissues, and in four cultured human cell lines (Coskun *et al.*, 2015). Extreme expression of MTH1 in malignant breast tumors was observed, suggesting that cancer cells are addicted to MTH1 for their survival. In agreement with this finding, cultured MCF-7 cells exhibited a much greater

expression level of hMTH1 than their normal counterpart mammary gland epithelial (MCF-10A) cells.

10. CONCLUSIONS

In this chapter, we describe the production and purification of full length ^{15}N-labeled DNA proteins to be used as internal standards for the measurement of DNA repair proteins in human tissues *in vivo* by LC-MS/MS or by other mass spectrometric techniques. These internal standards are absolutely essential for positive identification and accurate quantification of target proteins. The use of these labeled proteins has been demonstrated for the measurement of several DNA repair proteins in human tissues and cultured cells. Such measurements will be of fundamental importance for the determination of DNA repair capacity, the use of DNA repair proteins as biomarkers and the development of DNA repair inhibitors in cancer, and eventually in other diseases as well.

ACKNOWLEDGEMENTS

We are grateful to Dr. Dimitry Zharkov (SB RAS Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia) for providing the *ogg1* gene in the expression vector of pET15b for the production of ^{15}N-OGG1, and to Dr. Timothy R. O'Connor (Beckman Institute of the City of Hope, Duarte, CA) for the gift of the coding sequence for E. coli *fpg* gene from the plasmid pFpg for production of *E.coli* ^{15}N-Fpg. *E. coli* BL21 (λDE3) harboring pETApe recombinant of plasmid for the production of ^{15}N-hAPE1 was kindly provided by Dr. David M. Wilson III (National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA). The human *neil1* gene in the expression vector pET22b for the production of ^{15}N-hNEIL1 was a gift from Dr. Stephen Lloyd (Oregon Health and Science University, Portland, OR). This work on MTH1 protein was supported by the Knut and Alice Wallenberg Foundation (T.H.), Swedish Research Council (T.H.), Swedish Cancer Society (T.H.), the Swedish Pain Relief Foundation (T.H.), the Swedish Foundation for Strategic Research (T.H.), the Göran Gustafsson Foundation and the Torsten and Ragnar Söderberg Foundation (T.H.).

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or

endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

REFERENCES

Abbotts, R. & Madhusudan, S. (2010). Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. *Cancer Treatment Reviews, 36*, 425-435.

Aspinwall, R., Rothwell, D. G., Roldan-Arjona, T., Anselmino, C., Ward, C. J., Cheadle, J. P., Sampson, J. R., Lindahl, T., Harris, P. C., & Hickson, I. D. (1997). Cloning and characterization of a functional human homolog of *Escherichia coli* endonuclease III. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 109-114.

Audebert, M., Radicella, J. P., & Dizdaroglu, M. (2000). Effect of single mutations in the OGG1 gene found in human tumors on the substrate specificity of the ogg1 protein. *Nucleic Acids Research, 28*, 2672-2678.

Bandaru, V., Sunkara, S., Wallace, S. S., & Bond, J. P. (2002). A novel human DNA glycosylase that removes oxidative DNA damage and is homologous to Escherichia coli endonuclease VIII. *DNA Repair (Amst), 1*, 517-529.

Barnes, T., Kim, W. C., Mantha, A. K., Kim, S. E., Izumi, T., Mitra, S., & Lee, C. H. (2009). Identification of Apurinic/apyrimidinic endonuclease 1 (APE1) as the endoribonuclease that cleaves c-myc mRNA. *Nucleic Acids Research, 37*, 3946-3958.

Beard, W. A. & Wilson, S. H. (2000). Structural design of a eukaryotic DNA repair polymerase: DNA polymerase beta. *Mutation Research, 460*, 231-244.

Beard, W. A. & Wilson, S. H. (2006). Structure and mechanism of DNA polymerase Beta. *Chemical Reviews, 106*, 361-382.

Beard, W. A. & Wilson, S. H. (2014). Structure and mechanism of DNA polymerase beta. *Biochemistry, 53*, 2768-2780.

Beckman, R. A. & Loeb, L. A. (2005). Genetic instability in cancer: theory and experiment. *Seminars in Cancer Biology, 15*, 423-435.

Boiteux, S., Gajewski, E., Laval, J., & Dizdaroglu, M. (1992). Substrate specificity of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry, 31*, 106-110.

Braithwaite, D. K. & Ito, J. (1993). Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nucleic Acids Research, 21*, 787-802.

Cabelof, D. C. (2012). Haploinsufficiency in mouse models of DNA repair deficiency: modifiers of penetrance. *Cell Mol. Life Sci., 69*, 727-740.

Chan, M. K., Ocampo-Hafalla, M. T., Vartanian, V., Jaruga, P., Kirkali, G., Koenig, K. L., Brown, S., Lloyd, R. S., Dizdaroglu, M., & Teebor, G. W. (2009). Targeted deletion of the genes encoding NTH1

and NEIL1 DNA N-glycosylases reveals the existence of novel carcinogenic oxidative damage to DNA. *DNA Repair (Amst), 8*, 786-794.

Chan, N. & Bristow, R. G. (2010). "Contextual" synthetic lethality and/or loss of heterozygosity: tumor hypoxia and modification of DNA repair. *Clinical Cancer Research, 16*, 4553-4560.

Coskun, E., Jaruga, P., Jemth, A. S., Loseva, O., Scanlan, L. D., Tona, A., Lowenthal, M. S., Helleday, T., & Dizdaroglu, M. (2015). Addiction to MTH1 protein results in intense expression in human breast cancer tissue as measured by liquid chromatography-isotope-dilution tandem mass spectrometry. *DNA Repair (Ams),* (in press).

Curtin, N. J. (2013).*DNA Repair and Cancer From Bench to Clinic* , edited by S. Madhusudan & D. M. Wilson, III, pp. 513-563. Baco Raton: CRC Press.

Demple, B. & Harrison, L. (1994). Repair of oxidative damage to DNA: enzymology and biology. *Annual Review of Biochemistry, 63*, 915-948.

Demple, B., Herman, T., & Chen, D. S. (1991). Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes. *Proc. Natl. Acad. Sci. U. S. A, 88*, 11450-11454.

Dherin, C., Radicella, J. P., Dizdaroglu, M., & Boiteux, S. (1999). Excision of oxidatively damaged DNA bases by the human alpha-hOgg1 protein and the polymorphic alpha-hOgg1(Ser326Cys) protein which is frequently found in human populations. *Nucleic Acids Research, 27*, 4001-4007.

Dizdaroglu, M. (2005). Base-excision repair of oxidative DNA damage by DNA glycosylases. *Mutation Research, 591*, 45-59.

Dizdaroglu, M. (2015). Oxidatively induced DNA damage and its repair in cancer. *Mutat. Res. Rev. Mutat. Res., 763*, 212-245.

Dizdaroglu, M. & Jaruga, P. (2012). Mechanisms of free radical-induced damage to DNA. *Free Radical Research, 46*, 382-419.

Dizdaroglu, M., Reddy, P. T., & Jaruga, P. (2011). Identification and quantification of DNA repair proteins by liquid chromatography/isotope-dilution tandem mass spectrometry using their fully 15N-labeled analogues as internal standards. *Journal of Proteome Research., 10*, 3802-3813.

Doublie, S., Bandaru, V., Bond, J. P., & Wallace, S. S. (2004). The crystal structure of human endonuclease VIII-like 1 (NEIL1) reveals a zincless finger motif required for glycosylase activity. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 10284-10289.

Evans, M. D., Dizdaroglu, M., & Cooke, M. S. (2004). Oxidative DNA damage and disease: induction, repair and significance. *Mutation Research, 567*, 1-61.

Fishel, M. L., Vascotto, C., & Kelley, M. R. (2013).*DNA Repair and Cancer From Bench to Clinic* , edited by S. Madhusudan & D. M. Wilson, III, pp. 233-287. Baco Raton: CRC Press.

Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., & Ellenberger, T. (2006). *DNA Repair and Mutagenesis*, Second Edition ed. Washington, D.C.: ASM Press.

Gad, H., Koolmeister, T., Jemth, A. S., Eshtad, S., Jacques, S. A., Strom, C. E., Svensson, L. M., Schultz, N., Lundback, T., Einarsdottir, B. O., Saleh, A., Gokturk, C., Baranczewski, P., Svensson, R., Berntsson, R. P., Gustafsson, R., Stromberg, K., Sanjiv, K., Jacques-Cordonnier, M. C., Desroses, M., Gustavsson, A. L., Olofsson, R., Johansson, F., Homan, E. J., Loseva, O., Brautigam, L., Johansson, L., Hoglund, A., Hagenkort, A., Pham, T., Altun, M., Gaugaz, F. Z., Vikingsson, S., Evers, B., Henriksson, M., Vallin, K. S., Wallner, O. A., Hammarstrom, L. G., Wiita, E., Almlof, I., Kalderen, C., Axelsson, H., Djureinovic, T., Puigvert, J. C., Haggblad, M., Jeppsson, F., Martens, U., Lundin, C., Lundgren, B., Granelli, I., Jensen, A. J., Artursson, P., Nilsson, J. A., Stenmark, P., Scobie, M., Berglund, U. W., & Helleday, T. (2014). MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool. *Nature, 508*, 215-221.

Giglione, C., Boularot, A., & Meinnel, T. (2004). Protein N-terminal methionine excision. *Celullar Molecular Life Sciences, 61*, 1455-1474.

Gros, L., Ishchenko, A. A., Ide, H., Elder, R. H., & Saparbaev, M. K. (2004). The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway. *Nucleic Acids Research, 32*, 73-81.

Gros, L., Saparbaev, M. K., & Laval, J. (2002). Enzymology of the repair of free radicals-induced DNA damage. *Oncogene, 21*, 8905-8925.

Hadi, M. Z., Coleman, M. A., Fidelis, K., Mohrenweiser, H. W., & Wilson, D. M., III (2000). Functional characterization of Ape1 variants identified in the human population. *Nucleic Acids Research, 28*, 3871-3879.

Hakem, R. (2008). DNA-damage repair; the good, the bad, and the ugly. *EMBO Journal, 27*, 589-605.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *Journal of Molecular Biology, 166*, 557-580.

Hazra, T. K., Izumi, T., Boldogh, I., Imhoff, B., Kow, Y. W., Jaruga, P., Dizdaroglu, M., & Mitra, S. (2002). Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 3523-3528.

Hegde, M. L., Hazra, T. K., & Mitra, S. (2008). Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. *Cell Research, 18*, 27-47.

Helleday, T. (2008). Amplifying tumour-specific replication lesions by DNA repair inhibitors - a new era in targeted cancer therapy. *European Journal of Cancer, 44*, 921-927.

Helleday, T., Petermann, E., Lundin, C., Hodgson, B., & Sharma, R. A. (2008). DNA repair pathways as targets for cancer therapy. *Nature Reviews Cancer, 8*, 193-204.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature, 411*, 366-374.

Hu, J., de Souza-Pinto, N. C., Haraguchi, K., Hogue, B. A., Jaruga, P., Greenberg, M. M., Dizdaroglu, M., & Bohr, V. A. (2005). Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes. *Journal of Biological Chemistry, 280*, 40544-40551.

Huamani, J., McMahan, C. A., Herbert, D. C., Reddick, R., McCarrey, J. R., MacInnes, M. I., Chen, D. J., & Walter, C. A. (2004). Spontaneous mutagenesis is enhanced in Apex heterozygous mice. *Mol. Cell Biol., 24*, 8145-8153.

Huber, K. V., Salah, E., Radic, B., Gridling, M., Elkins, J. M., Stukalov, A., Jemth, A. S., Gokturk, C., Sanjiv, K., Stromberg, K., Pham, T., Berglund, U. W., Colinge, J., Bennett, K. L., Loizou, J. I., Helleday, T., Knapp, S., & Superti-Furga, G. (2014). Stereospecific targeting of MTH1 by (S)-crizotinib as an anticancer strategy. *Nature, 508*, 222-227.

Illuzzi, J. L. & Wilson, D. M., III (2012). Base excision repair: contribution to tumorigenesis and target in anticancer treatment paradigms. *Current Medicinal Chemistry, 19*, 3922-3936.

Jaruga, P., Birincioglu, M., Rosenquist, T. A., & Dizdaroglu, M. (2004). Mouse NEIL1 protein is specific for excision of 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine from oxidatively damaged DNA. *Biochemistry, 43*, 15909-15914.

Jaruga, P., Xiao, Y., Vartanian, V., Lloyd, R. S., & Dizdaroglu, M. (2010). Evidence for the involvement of DNA repair enzyme NEIL1 in nucleotide excision repair of (5'*R*)- and (5'*S*)-8,5'-cyclo-2'-deoxyadenosines. *Biochemistry, 49*, 1053-1055.

Karahalil, B., Roldan-Arjona, T., & Dizdaroglu, M. (1998). Substrate specificity of *Schizosaccharomyces pombe* Nth protein for products of oxidative DNA damage. *Biochemistry, 37*, 590-595.

Kelley, M. R. (2012).*DNA Repair in Cancer Therapy, Molecular Targets and Clinical Applications* , edited by M. R. Kelley, pp. 301-310. Amsterdam: Elsevier.

Kennedy, C. H., Cueto, R., Belinsky, S. A., Lechner, J. F., & Pryor, W. A. (1998). Overexpression of hMTH1 mRNA: a molecular marker of oxidative stress in lung cancer cells. *FEBS Letters, 429*, 17-20.

Kinter, M. & Sherman, N. E. (2000). *Protein Sequencing and Identification Using Tandem Mass Spectrometry*. New York: Wiley-Interscience.

Kirkali, G., Jaruga, P., Reddy, P. T., Tona, A., Nelson, B. C., Li, M., Wilson, D. M., III, & Dizdaroglu, M. (2013). Identification and quantification of DNA repair protein apurinic/apyrimidinic endonuclease 1 (APE1) in human cells by liquid chromatography/isotope-dilution tandem mass spectrometry. *PLoS ONE, 8*, e69894.

Liu, M., Bandaru, V., Bond, J. P., Jaruga, P., Zhao, X., Christov, P. P., Burrows, C. J., Rizzo, C. J., Dizdaroglu, M., & Wallace, S. S. (2010). The mouse ortholog of NEIL3 is a functional DNA

glycosylase in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 4925-4930.

Liu, R., Yin, L. H., & Pu, Y. P. (2007). Reduced expression of human DNA repair genes in esophageal squamous-cell carcinoma in china. *J. Toxicol. Environ. Health A, 70*, 956-963.

Loeb, L. A. (2011). Human cancers express mutator phenotypes: origin, consequences and targeting. *Nature Reviews Cancer, 11*, 450-457.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265-275.

Madhusudan, S. & Middleton, M. R. (2005). The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer. *Cancer Treatment Reviews, 31*, 603-617.

Maki, H. & Sekiguchi, M. (1992). MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature, 355*, 273-275.

Matsumoto, Y. & Kim, K. (1995). Excision of deoxyribose phosphate residues by DNA polymerase β during DNA repair. *Science, 269*, 699-702.

Meira, L. B., Devaraj, S., Kisby, G. E., Burns, D. K., Daniel, R. L., Hammer, R. E., Grundy, S., Jialal, I., & Friedberg, E. C. (2001). Heterozygosity for the mouse Apex gene results in phenotypes associated with oxidative stress. *Cancer Research, 61*, 5552-5557.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mo, J. Y., Maki, H., & Sekiguchi, M. (1992). Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 11021-11025.

Moeller, B. J., Arap, W., & Pasqualini, R. (2010). Targeting synthetic lethality in DNA damage repair pathways as an anti-cancer strategy. *Currunt Drug Targets, 11*, 1336-1340.

Mohrenweiser, H. W., Xi, T., Vazquez-Matias, J., & Jones, I. M. (2002). Identification of 127 amino acid substitution variants in screening 37 DNA repair genes in humans. *Cancer Epidemiology, Biomarkers and Prevention, 11*, 1054-1064.

Muftuoglu, M., de Souza-Pinto, N. C., Dogan, A., Aamann, M., Stevnsner, T., Rybanska, I., Kirkali, G., Dizdaroglu, M., & Bohr, V. A. (2009). Cockayne syndrome group B protein stimulates repair of formamidopyrimidines by NEIL1 DNA glycosylase. *Journal of Biological Chemistry, 284*, 9270-9279.

O'Connor, T. R., Boiteux, S., & Laval, J. (1989). Repair of imidazole ring-opened purines in DNA: overproduction of the formamidopyrimidine-DNA glycosylase of Escherichia coli using plasmids containing the fpg+ gene. *Ann. Ist. Super. Sanita, 25*, 27-31.

Okamoto, K., Toyokuni, S., Kim, W. J., Ogawa, O., Kakehi, Y., Arao, S., Hiai, H., & Yoshida, O. (1996). Overexpression of human mutT homologue gene messenger RNA in renal-cell carcinoma: evidence of persistent oxidative stress in cancer. *International Journal of Cancer, 65*, 437-441.

Perry, C., Sultana, R., & Madhusudan, S. (2012).*DNA Repair in Cancer Therapy: Molecular Targets and Clinical Applications* , edited by M. R. Kelley, pp. 257-282. Amsterdam: Elsevier.

Pieretti, M., Khattar, N. H., & Smith, S. A. (2001). Common polymorphisms and somatic mutations in human base excision repair genes in ovarian and endometrial cancers. *Mutation Research, 432*, 53-59.

Reddy, P. T., Jaruga, P., Kirkali, G., Tuna, G., Nelson, B. C., & Dizdaroglu, M. (2013). Identification and quantification of human DNA repair Protein NEIL1 by liquid chromatography/isotope-dilution tandem mass spectrometry. *Journal of Proteome Research., 12*, 1049-1061.

Reddy, P. T., Jaruga, P., Nelson, B. C., Lowenthal, M., & Dizdaroglu, M. (2011). Stable isotope-labeling of DNA repair proteins, and their purification and characterization. *Protein Expression and Purification, 78*, 94-101.

Roldán-Arjona, T., Anselmino, C., & Lindahl, T. (1996). Molecular cloning and functional analysis of a *Schizosaccharomyces pombe* homologue of *Escherichia coli* endonuclease III. *Nucleic Acids Research, 24*, 3307-3312.

Rosenquist, T. A., zaika, E., Fernandes, A. S., Zharkov, D. O., Miller, H., & Grollman, A. P. (2003). The novel DNA glycosylase, NEIL1, protects mammalian cells from radiation-mediated cell death. *DNA Repair (Amst), 2*, 581-591.

Roy, L. M., Jaruga, P., Wood, T. G., McCullough, A. K., Dizdaroglu, M., & Lloyd, R. S. (2007). Human polymorphic variants of the NEIL1 DNA glycosylase. *Journal of Biological Chemistry, 282*, 15790-15798.

Sakai, Y., Furuichi, M., Takahashi, M., Mishima, M., Iwai, S., Shirakawa, M., & Nakabeppu, Y. (2002). A molecular basis for the selective recognition of 2-hydroxy-dATP and 8-oxo-dGTP by human MTH1. *Journal of Biological Chemistry, 277*, 8579-8587.

Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., & Sekiguchi, M. (1993). Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *Journal of Biological Chemistry, 268*, 23524-23530.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sampath, H., McCullough, A. K., & Lloyd, R. S. (2012). Regulation of DNA glycosylases and their role in limiting disease. *Free Radical Research, 46*, 460-478.

Sekiguchi, M. & Tsuzuki, T. (2002). Oxidative nucleotide damage: consequences and prevention. *Oncogene, 21*, 8895-8904.

Sidorenko, V. S., Grollman, A. P., Jaruga, P., Dizdaroglu, M., & Zharkov, D. O. (2009). Substrate specificity and excision kinetics of natural polymorphic variants and phosphomimetic mutants of human 8-oxoguanine-DNA glycosylase. *FEBS J., 276*, 5149-5162.

Speina, E., Arczewska, K. D., Gackowski, D., Zielinska, M., Siomek, A., Kowalewski, J., Olinski, R., Tudek, B., & Kusmierek, J. T. (2005). Contribution of hMTH1 to the maintenance of 8-oxoguanine levels in lung DNA of non-small-cell lung cancer patients. *J Natl. Cancer Inst., 97*, 384-395.

Srivastava, D. K., Berg, B. J., Prasad, R., Molina, J. T., Beard, W. A., Tomkinson, A. E., & Wilson, S. H. (1998). Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps. *Journal of Biological Chemistry, 273*, 21203-21209.

Svensson, L. M., Jemth, A. S., Desroses, M., Loseva, O., Helleday, T., Hogbom, M., & Stenmark, P. (2011). Crystal structure of human MTH1 and the 8-oxo-dGMP product complex. *FEBS Letters, 585*, 2617-2621.

Tell, G., Quadrifoglio, F., Tiribelli, C., & Kelley, M. R. (2009). The many functions of APE1/Ref-1: not only a DNA repair enzyme. *Antioxidants Redox Signalling, 11*, 601-620.

Tsuzuki, T., Egashira, A., Igarashi, H., Iwakuma, T., Nakatsuru, Y., Tominaga, Y., Kawate, H., Nakao, K., Nakamura, K., Ide, F., Kura, S., Nakabeppu, Y., Katsuki, M., Ishikawa, T., & Sekiguchi, M. (2001). Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 11456-11461.

Vartanian, V., Lowell, B., Minko, I. G., Wood, T. G., Ceci, J. D., George, S., Ballinger, S. W., Corless, C. L., McCullough, A. K., & Lloyd, R. S. (2006). The metabolic syndrome resulting from a knockout of the NEIL1 DNA glycosylase. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 1864-1869.

Wallace, S. S., Murphy, D. L., & Sweasy, J. B. (2012). Base excision repair and cancer. *Cancer Letters, 327*, 73-89.

Wilson, D. M., III & Barsky, D. (2001). The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. *Mutation Research, 485*, 283-307.

Wilson, D. M., III, Kim, D., Berquist, B. R., & Sigurdson, A. J. (2011). Variation in base excision repair capacity. *Mutation Research, 711*, 100-112.

Wood, R. D., Mitchell, M., & Lindahl, T. (2005). Human DNA repair genes. *Mutation Research, 577*, 275-283.

Xanthoudakis, S. & Curran, T. (1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO Journal, 11*, 653-665.

Xanthoudakis, S., Smeyne, R. J., Wallace, J. D., & Curran, T. (1996). The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 8919-8923.

FIGURE CAPTIONS

Figure 1. SDS (13 %)-PAGE analysis of the purification steps of *E. coli* ^{15}N-Fpg: Lane 1, 21 µg protein of the extract of BL21(DE3) cells harboring pET11a control plasmid; Lane 2, 31 µg protein of the extract of BL21(DE3) cells harboring Fpg/pET11a plasmid induced with IPTG; Lane 3, 26 µg of 10000 x g supernatant; Lane 4, 8.6 µg flow through from DEAE cellulose column, 29 µg; Lane 5, 8.5 µg Fpg eluted from CM cellulose column, 26 µg; Lane 6, Molecular mass markers. (data from (Reddy *et al.*, 2011)).

Figure 2: SDS (10 %)-PAGE analysis of the purification steps of ^{15}N-hOGG1: Lane 1, 20 µg protein of the extract of BL21(DE3) cells harboring pET11a control plasmid; Lane 2, 21 µg protein of the extract of BL21(DE3) cells harboring hOGG1/pET15b plasmid induced with IPTG; Lane 3, 17 µg of 48000 x g supernatant; Lane 4, 15.5 µg from Ni-agarose flow through; Lane 5, 8.3 µg hOGG1 eluted from Ni-agarose column; Lane 6, Molecular mass markers. (data from (Reddy *et al.*, 2011))

Figure 3: SDS (10 %)-PAGE analysis of the purification steps of ^{15}N-hNEIL1: Lane 1, Uninduced cell extract, 24 µg; Lane 2, Induced cell extract, 28 µg; Lane 3, 20000 x g supernatant, 22 µg; Lane 4, Flow through from Nickel resin, 17 µg; Lane 5, Pure hNEIL1 eluted from Nickel resin, 15 µg; Lane 6, Molecular mass markers. (data from (Reddy *et al.*, 2013)).

Figure 4: SDS (11 %)-PAGE analysis of the purification steps of ^{15}N-hAPE1: Lane 1, Uninduced cell extract, 24 µg; Lane 2, Induced cell extract, 28 µg; Lane 3, 70000 x g supernatant, 24 µg; Lane 4, Flow through from DEAE cellulose column, 17 µg; Lane 5, Flow through from CM cellulose column, 16 µg; Lane 6, Pure hNEIL1 eluted from CM cellulose column, 21 µg; Lane 7, Molecular mass markers. (data from (Kirkali *et al.*, 2013)).

Figure 5: SDS-PAGE analysis of purified hMTH1 and ^{15}N-hMTH1. Lane 1, hMTH1; Lane 2, ^{15}N-hMTH1; Lane 7, Molecular mass markers (data from (Coskun *et al.*, 2015)).

Figure 6: Full-scan mass spectra of the tryptic peptides GAVAEDGDELR (**A**) and ^{15}N-GAVAEDGDELR (**B**). (data from (Kirkali *et al.*, 2013)).

Figure 7: Product ion spectra of GAVAEDGDELR (A) and ^{15}N-GAVAEDGDELR (B). The $(M+2H)^{2+}$ ions *m/z* 566.3 (**A**) and *m/z* 573.3 (**B**) were used as the precursor ions. The insert shows the fragmentation pathways leading to the *b*- and *y*-ions. (data from (Kirkali *et al.*, 2013)).

Figure 8: Ion-current profiles of mass transitions of five tryptic peptides of hAPE1 and ^{15}N-hAPE1 obtained using the tryptic digest of a nuclear extract of MCF-7 cells. The extract was spiked with an aliquot of ^{15}N-hAPE1 prior to separation by HPLC. (data from (Kirkali *et al.*, 2013)).