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New Players in Recognition of Intact and Cleaved AP Sites: Implication in DNA Repair in Mammalian Cells

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1. Introduction

The apurinic/apyrimidinic (AP) sites (called also abasic sites) are common lesion in genomic DNA, arising at a frequency of 10,000 to 50,000 lesions per mammalian cell per day (Lindahl, 1993). Unrepaired AP sites present mutagenic and cytotoxic consequences to the cell (Wilson; & Thompson, 1997). Most of the abasic sites are believed to result directly from spontaneous depurination, or indirectly from deamination of cytosine to uracil, which is then eliminated by uracil glycosylases. AP sites also result from hydrolysis of oxidized or alkylated bases by lesion-specific glycosylases during the early stage of base excision repair (BER) (McCullough et al., 1999). AP sites in isolated DNA are rather stable, but can be converted to single-strand breaks by alkali treatment, heating or nucleophilic attack at the aldehydic C1' group (Burrows & Muller, 1998). Intact abasic sites are noncoding lesions and *in vivo* can be stable enough to be mutagenic during DNA replication (Loeb & Preston, 1986). To protect genome integrity, eukaryotic organisms have robust enzyme activities, mainly APE1 in mammalian cells (Wilson & Barsky, 2001), that recognize abasic sites and cut the DNA backbone initiating the repair process. The continuous generation and repair of AP sites results in a steady-state levels of AP sites in mammalian cells in the range of approximately 1 site per 10^6 nucleotides (Atamna et al., 2000, Mohsin Ali et al., 2004). The number of AP sites can increase dramatically under stressful conditions such as X-ray or UV light irradiation or oxidative and alkylating agent exposure (Atamna et al., 2000). Considering the ubiquity of these lesions, it is reasonable to assume that wide range of cellular proteins can interact with abasic sites depending on the physiological state and stages of cell cycle.

The chapter is devoted to search of previously unrecognized proteins capable to interact with intact or cleaved AP sites. We mainly focused on proteins that form Schiff base upon this interaction. In most cases, these proteins are able to process AP sites although less efficiently than previously known counterparts. The biological role of these interactions in providing of backup pathways of DNA repair processes is also discussed.

2. Proteins that recognize AP sites

In genomic DNA of higher eukaryotes AP sites irrespectively of their origin are thought to be repaired by base excision repair (Almeida & Sobol, 2007; Hegde et al., 2008).

2.1 Base excision repair (overview)

Base excision repair (BER) is one of the major systems of DNA repair, mostly responsible for removing from DNA of non-bulky base lesions that appear in the genome with high frequency (Almeida & Sobol, 2007; Hegde et al., 2008; Schärer, 2003). The base excision repair pathway essentially removes and replaces nucleotides containing aberrant bases in DNA. Metabolically produced reactive nitrogen and oxygen species can modify the DNA bases due to oxidation, deamination and even alkylation at several positions in the base. BER is usually defined as DNA repair initiated by a lesion-specific DNA glycosylase and completed by either of two sub-pathways: short-patch BER; a mechanism whereby only 1 nucleotide is replaced or long-patch BER; a mechanism whereby 2–13 nucleotides are replaced (Frosina et al., 1996; Klungland & Lindahl, 1997).

The majority of BER is currently thought to occur via the short-patch pathway initiated by either a mono-functional or bi-functional DNA glycosylases. The short-patch BER pathway mediated by a mono-functional glycosylase involves removal of aberrant base by the lesion-specific DNA glycosylases, enzymes that hydrolyze cleavage of *N*-glycosylic bond between base and deoxyribose. This results in apurinic/apyrimidinic (also termed abasic) sites (AP sites), which repair is generally initiated through strand incision at its 5'-side by an AP endonuclease, leaving a nick flanked by a 3'-hydroxyl of an undamaged and a deoxyriboso-5'-phosphate (dRP), to which the damaged base was formerly linked. DNA polymerase then inserts a normal deoxyribonucleotide; however, the ligation step to restore intact DNA is blocked because of the dangling dRP moiety. The situation is resolved by a special enzymatic activity, excising dRP moiety (short-patch), or by continuing DNA synthesis with strand displacement, followed by removal of the displaced strand by flap endonuclease 1 (long-patch BER) (Frosina et al., 1996; Klungland & Lindahl, 1997).

In mammalian cells, the major dRP-removing enzyme is DNA polymerase β (Pol β), a multifunctional enzyme consisting of the 8-kDa amino-terminal domain with deoxyribose phosphate (dRP) lyase activity and the 31-kDa carboxy-terminal domain with nucleotidyl transferase activity (Allinson et al., 2001, Matsumoto & Kim, 1995, Piersen et al., 1996, Podlutsky et al., 2001). Thus, Pol β can mediate both steps in single nucleotide BER: insertion of deoxyribonucleotide and removal of dRP moiety preparing the strand for ligation.

Oxidized bases in DNA are manly produced by reactive oxygen species (ROS). ROS are generated as by-products of metabolic processes, primarily oxidative metabolism in the mitochondria (Dawson et al., 1993), and pathological conditions such as inflammation. ROS are also generated by ionizing radiation and some chemotherapeutic drugs (Almeida & Sobol, 2007; Hegde et al., 2008; Schärer, 2003). Oxidized bases are primarily removed by bi-functional DNA glycosylases that have an additional AP site cleavage activity. In addition to oxidation of DNA bases, ROS attack deoxyribose in DNA to generate strand breaks with nonligatable ends. The 3′ blocking groups include 3′ phosphate, 3′ phosphoglycolaldehyde, or 3′ phosphoglycolate (Breen et al., 1995). The 5′ terminus normally contains phosphate but after ROS reaction the nonligatable ends include 5′ OH and 5′ phosphodeoxyribose derivatives such as 2-deoxyribonolactone (Demple et al., 2002). Repair of single-strand breaks (SSBR) with blocked termini utilizes many of the same proteins as the BER process. The principal difference between SSBR and BER is the initiation step. Further both processes may occur at the single nucleotide level (i.e., short-patch repair) or as a long-patch of repair. BER is initiated via DNA glycosylase activity that results in the removal of a damaged base. SSBR is defined specifically for the repair of single-strand breaks in DNA generated by

irradiation or ROS (Fan & Wilson, 2005). AP sites appeared via spontaneous base loss and arising from irradiation are also further processed by the BER machinery.

2.2 Processing of AP sites

AP sites can be incised by three mechanistically different ways represented in Fig.1.

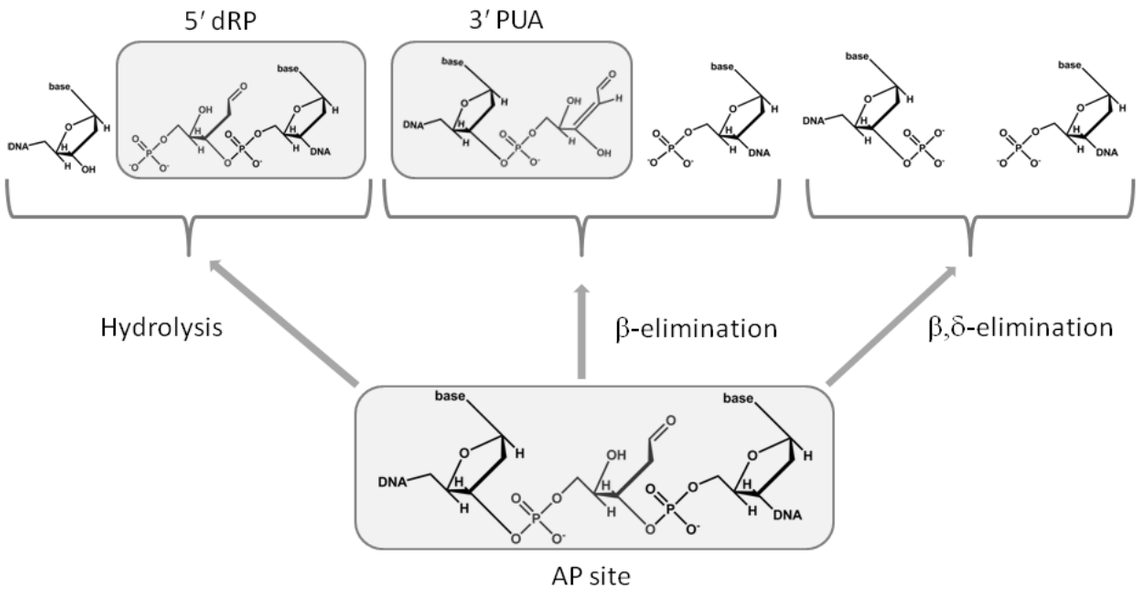

Fig. 1. Structures of intact and cleaved AP sites. DNA structures containing reactive sugar moieties capable of Schiff base formation are marked by grey rectangular boxes.

In mammalian cells, the repair of AP sites is generally initiated through strand incision by APE1, the second enzyme of the canonical BER pathway (Demple et al., 1991). APE1 is thought to process of over 95% of AP sites in mammalian cells (Demple & Harrison, 1994; Wilson & Barsky, 2001). APE1 catalyzes hydrolysis of AP sites by a Mg^{2+}-dependent mechanism, resulting in cleavage of the phosphodiester bond 5′ to the AP site and generation of a single-strand break. This reaction produces a 3′-hydroxyl group and a 5′-dRP group flanking the break. This way of AP site processing fits short-patch pathway of the BER process.

Another way of AP site cleavage by the BER machinery involving the action of bi-functional DNA glycosylases is more complicated and requires the 'end cleaning' of termini prior to the further repair reactions may occur. An intermediate reaction step for these enzymes is the formation of a transient Schiff base between the amino group and the C1′ of deoxyribose for both base excision and subsequent DNA strand cleavage. In solution, AP sites exist predominantly as a mixture of ring-closed α- and β-hemiacetals with a minor amount of ring-opened aldehyde and aldehyde hydrate (<1%) (de los Santos et al., 2004). AP sites can be incised through β-elimination using ε-NH2 of a lysine as the active site nucleophile (David & Williams, 1998; McCullough et al., 1999). Strand incision by β-elimination forms a nick with phospho-α,β-unsaturated aldehyde (PUA) at the 3′ margin and phosphate at the 5′ margin (David & Williams, 1998; McCullough et al., 1999) (Fig. 1). While recently discovered mammalian DNA glycosylases, termed NEIL (Nei-like)-1, -2, catalyze the β,δ-elimination

reaction with the N-terminal proline being used as the nucleophile (Hazra et al., 2002; Takao et al., 2002, Das et al., 2006). The β,δ-elimination reaction results in a one nucleotide gap flanked with phosphate groups at the 3′- and 5′ margins. The products of bi-functional DNA glycosylase activity require the trimming of the 3′-ends to produce 3′-end hydroxyl groups that are indispensable for DNA polymerase activity.

Most of mammalian proteins known to form the Schiff base intermediate with the AP site appear to belong to the BER system. On the other hand, an interaction of AP DNA with proteins not formally involved in BER (for example, human ribosomal protein S3 and nucleoside diphosphate kinase – NM23-H2/NDP) has been well documented (Hegde et al., 2004; Postel et al., 2000). These two proteins are able to cleave AP sites. MutY, unlike other monofuctional DNA glycosylases, is able to interact with AP site via a Schiff base formation (Zharkov & Grollman, 1998) without the concomitant cleavage of AP sites. In this particular case formation of the Schiff base intermediate is considered as a mechanism for temporal protection of AP sites (Zharkov & Grollman, 1998).

Since AP sites in DNA appear to be promiscuous in their binding to many cellular factors and they are constantly generated in high frequency in genomic DNA, it may be important to sequester the AP sites immediately upon formation and further process them by the way that is most favorable for DNA integrity. Thus, interaction of cellular proteins with AP sites might be important for their repair/temporal protection from further degradation or might be involved in damage sensing/signaling making search of AP site reactive proteins a very important task.

2.3 Search of new participants of recognition/processing of AP sites

The very promising approach in search for unknown players in AP site recognition is based on a well-known propensity of deoxyribose in AP site, existing in equilibrium between cyclic furanose and acyclic aldehyde forms, to react with amine moieties in its vicinity. The Schiff base intermediate can be reduced by sodium borohydride ($NaBH_4$) or related compounds, forming an irreversible complex between the enzyme and DNA (David & Williams, 1998; Piersen et al., 2000). This reaction is widely used to prove the β-elimination reaction mechanism for the enzymes capable of AP site cleavage (David & Williams, 1998; Piersen et al., 2000), although some proteins (e.g. MutY DNA glycosylase) can form a Schiff base with no further β-elimination (Zharkov & Grollman, 1998). Therefore, upon searching of new players in AP site recognition mediated by the Schiff base formation their ability to cleave AP has to be proved. Along with intact AP site, the product of its cleavage – 3′ PUA and 5′ dRP moieties (Fig. 1, grey rectangular boxes) are also able to form the Schiff base intermediate with primary amino groups of proteins that allows to use these DNA in search and identification of proteins.

2.3.1 Identification of proteins reactive to AP sites

For identification of protein cross-linked to AP DNA in cell extracts immunochemical and/or mass-spectrometry methods can be used. Identification of proteins reactive to AP sites by combination of cross-linking technique and mass-spectrometry is schematically represented in Fig. 2.

In general, search and identification of proteins reactive to AP sites include the following steps: (1) finding of cellular extract proteins forming covalent adducts with AP sites in DNA; (2) the design of AP-DNA probe containing a functional group providing the selective

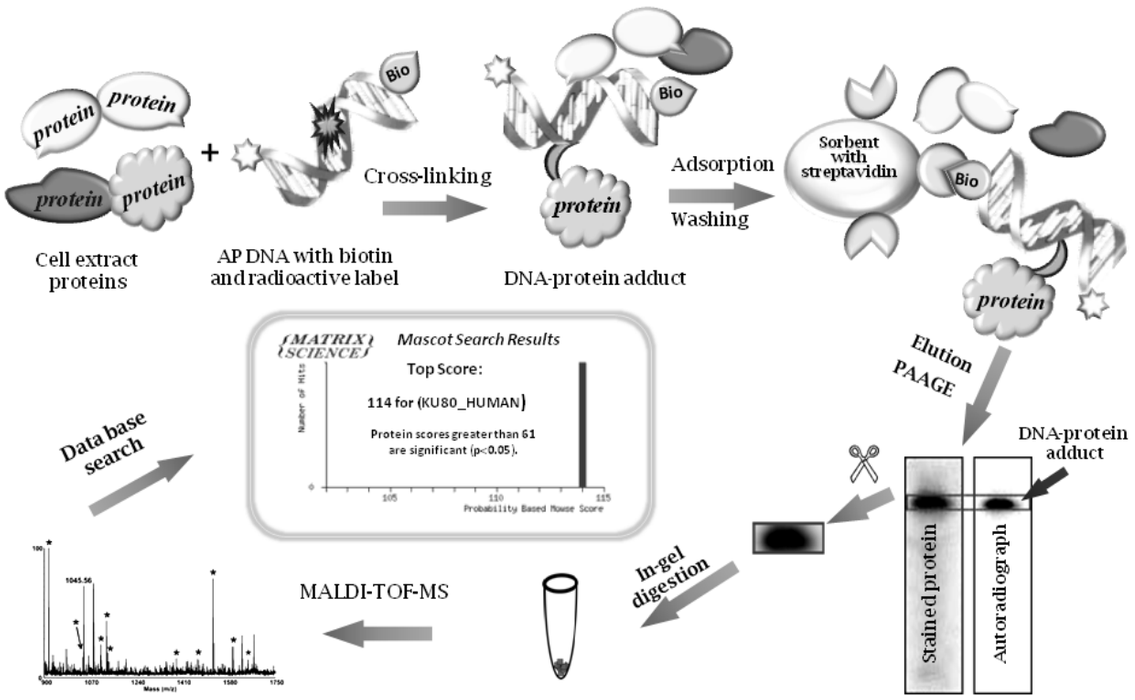

Fig. 2. Workflow of identification of protein cross-linked to DNA containing intact or cleaved AP site.

isolation of cross-linked DNA-protein products; (3) the preparative cross-linking of extract proteins with such DNA; (4) the affinity purification of the resulted products; (5) the identification of the protein in the covalent adduct with DNA using mass-spectrometry methods; (6) the confirmation of the results using purified proteins and/or specific antibodies and the known functions/interactions of the identified protein; and (7) the study of the functional role of the revealed interactions between proteins and AP DNA. A proof-of-principle identification of protein reactive towards AP sites in species from *E. coli* and *S. cerevisiae* was reported in (Rieger et al., 2006).

It should be noted that used protocol includes two steps that increases the selectivity of the method. Affinity purification of cross-linked protein was carried out on commercially available affinity sorbents containing streptavidin, which binds the biotin residue. After adsorption, non-specifically bound proteins were removed by a series of washings. Additional separation of proteins was achieved by electrophoresis under denaturing conditions by the Laemmli method (Laemmli, 1970) followed by staining proteins. The target product was identified by the colocalization in the gel positions of the label in DNA and the stained protein. This approach indicates that only the protein cross-linked to DNA will be subjected to the further analysis. However, one cannot fully exclude the presence of impurity proteins with the same electrophoretic mobility as the target product.

The identification was carried out for a number of proteins forming cross-links with AP sites in extracts of *E. coli* cells at logarithmic and stationary phases of cell growth (Rieger et al., 2006). It should be noted that authors used the previously developed non-enzymatic approach of the creation of AP sites in DNA based on the periodate oxidation of 2,3,5,6-tetrahydroxyhexyl phosphate precursor, which was introduced into an oligonucleotide

during the standard phosphoroamidite oligonucleotide synthesis. The periodate oxidation allows one to obtain AP sites in an almost quantitative yield. This method, in the authors' opinion, has several advantages over the more commonly used method that is based on the removal of uracil residues using uracil DNA glycosylase.

The procaryotic proteins AroF, DnaK, MutM, PolA, TnaA, TufA, and UvrA from *E. coli* and eucaryotic ARC1 and Ygl245wp from yeast were identified (Rieger et al., 2006). Protein Ygl245wp with an unknown but essential for cell viability function was prepared in the individual state after its coding sequence was cloned, and the recombinant plasmid was used for the production of the protein in *E. coli* cells followed by its isolation. The obtained protein was shown to bind to AP DNA forming Schiff bases; however, the biological significance of this interaction has not been established. DNA polymerase I, like other DNA polymerases of the A-family, has a weak AP lyase activity and dRP lyase activity (Pinz & Bogenhagen, 2000). However the biological significance of these activities *in vivo* has not been established. The ability of UvrA, the key component of the nucleotide excision repair (NER) in bacteria, to interact with AP sites in the UvrABC complex indicates the possible role of NER as a back-up pathway of AP site repair in bacteria (Showden et al., 1990).

2.3.2 Identification of Ku80 subunit of human Ku antigen as a protein reactive to AP sites

Using the same technique we screened mammalian cell extracts for proteins capable of binding AP site-containing DNA. In HeLa cell extract, a 32-bp DNA duplex with an AP site in the middle of the DNA chain was shown to cross-link predominantly to a protein forming a product with an apparent molecular mass of 95 kDa (Fig. 3) (Ilina et al., 2008).

The subsequent analysis of whole cell extracts of human lung fibroblasts, K-562, and MCF-7 cells revealed the products with the same electrophoretic mobility. The preferential cross-linking of this protein reflected extraordinary specificity in light of the multitude of proteins in the cell. If the AP site in DNA is replaced by its analog (the THF residue) or the treatment with NaBH4 is omitted, DNA–protein cross-linked products are not registered (Fig. 3).

In spite of efficient cross-linking of the above mentioned AP DNA to Ku80 polypeptide (as a part of DNA-PK) no cleavage of AP sites was observed (Ilina et al., 2008). Instead Ku80 formed with AP site a long-living Schiff base intermediate without the concomitant AP site cleavage just as was observed for monofunctional DNA glycosylase MutY and considered as a mechanism for temporal protection of AP sites (Zharkov & Grollman, 1998). AP lyase and 5′-dRP activities are distinctive features of the BER process (Almeida & Sobol, 2007; Hegde et al., 2008). The BER 5′-dRP/AP lyases usually function beyond the DS-breaks. But abasic sites associated with double-strand breaks can be generated by ionizing radiation, by treatment with radiomimetic drugs or as a result of attempted BER of complex damages (Yang et al., 2004). These specifically positioned lesions must be removed prior to or in the course of DS break repair. Ku antigen – DS-end binding protein of NHEJ – has been recently shown to act as a 5′-dRP/AP lyase near double-strand breaks (Roberts et al., 2010).

At DSB ends Ku is approximately tenfold more active than Pol β (Roberts et al., 2010), the most known mammalian 5′-dRP lyase (Matsumoto & Kim, 1995). At the same time, Ku was inefficient as AP lyase at AP sites situated at a distance longer than one helix turn from DS breaks just as was reported in (Ilina et al., 2008). $NaBH_4$-dependent cross-linking of

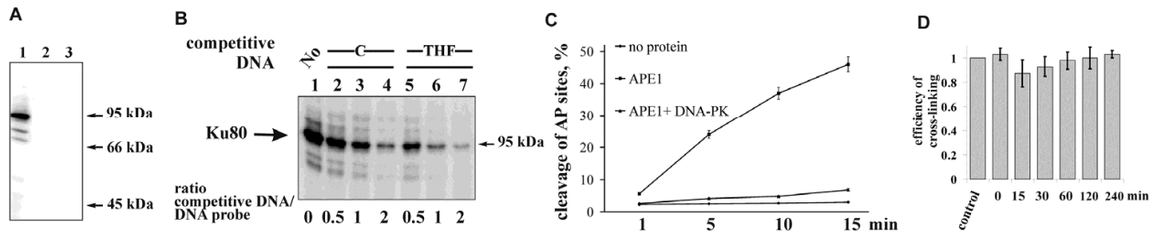

Fig. 3. Interaction of Ku antigen with AP sites (From Ilina et al., 2008). (A) Cross-linking of proteins in HeLa cell extract (lane 1); without borohydride treatment (lane 2); AP DNA probe was replaced by the DNA duplex containing a THF residue (lane 3). (B) Specificity of the Ku80 antigen interaction with AP DNA. Cross-linking of the HeLa cell extract proteins to AP DNA was performed in the absence (lane 1) or presence of competitive DNA at different concentrations (lanes 2-7). The structures of competitive DNAs are shown at the top. Ratio of competitive DNA to DNA probe is shown at the bottom. (C) Influence of DNA-PK on the activity of APE1. (D) Estimation of the stability of Ku complex with AP DNA in HeLa cell extract. AP DNA was preincubated with HeLa cell extract for 15 min at 37°. Then excess of competitive DNA containing a THF residue was added, and the reaction mixture was further incubated at 37° for additional 4 hours. Aliquots at different times were reduced with $NaBH_4$ and analyzed.

appropriate AP lyase substrate and AP lyase activity test in the cell extracts deficient and proficient in Ku antigen unambiguously testify to the role of Ku antigen in processing of AP sites positioned near 5′ termini of DS breaks. Moreover, transfection of Ku deficient or proficient cells with variants of specifically designed substrate DNAs (with natural AP site or its AP lyase-resistant analog or without AP site) followed by PCR amplification of joining products and subsequent restriction analysis of amplicons fully confirmed the necessity of Ku antigen AP lyase activity for removal of near-end AP sites. Altogether the results obtained *in vitro* and *in vivo* testify to use of the 5′-dRP/AP lyase activity of Ku antigen for the excision of near-end abasic sites and explain higher radiosensitivity of mammalian cells deficient in Ku antigen, which is indispensable for classical NHEJ (Schulte-Uentrop et al., 2008). It is worthy of notice, that the same mechanism of AP site cleavage is used by two unrelated DNA repair systems and the suitable positioning of AP sites relative to active site nucleophiles is indispensable for efficient catalysis.

2.3.3 AP site recognition by the 5'-dRP/AP lyase in PARP-1

In further screening for proteins that are reactive to AP sites in addition to a linear DNA duplex with an AP site in the middle of the ^{32}P-5′end-labeled strand, we used circular AP site-containing DNA to exclude interference by Ku80. Circular double-stranded DNA was synthesized, using single-stranded M13 DNA as template, in the presence of dUTP; then, AP sites were generated by uracil DNA glycosylase treatment (Khodyreva et al., 2010a; Khodyreva et al., 2010b). Unlike short duplex DNA with an AP site, that predominantly cross-linked Ku80 in HeLa cell extract (Ilina et al., 2008 and Fig. 4A, lane 5), the use of circular AP site-containing DNA allowed us to detect a novel protein with molecular mass of ~120-kDa that is reactive to AP site (Fig. 4A, lanes 1–4).

To identify the cross-linked protein large-scale cross-linking with the bovine testis nuclear extract (BTNE) and a biotin-containing linear AP DNA was performed. Identification was realized according to the scheme shown in Fig. 2.

Results from the MS analyses were searched against a database, and PARP-1 was identified as the first-rank candidate (Mascot probability score of 248, 38% of coverage).
We tested for and found AP site cross-linking by purified PARP-1 (data not shown). Yet, it was not clear whether the cross-linked complex in the extract resulted from PARP-1's reactivity at the intact AP site or a pre-incised AP site.
We next examined purified PARP-1 cross-linking with a linear DNA containing either an intact AP site or pre-incised AP site; in addition to cross-linking probes, these DNAs are substrates for 5'-dRP and AP lyase enzymatic activity (Fig. 4B).

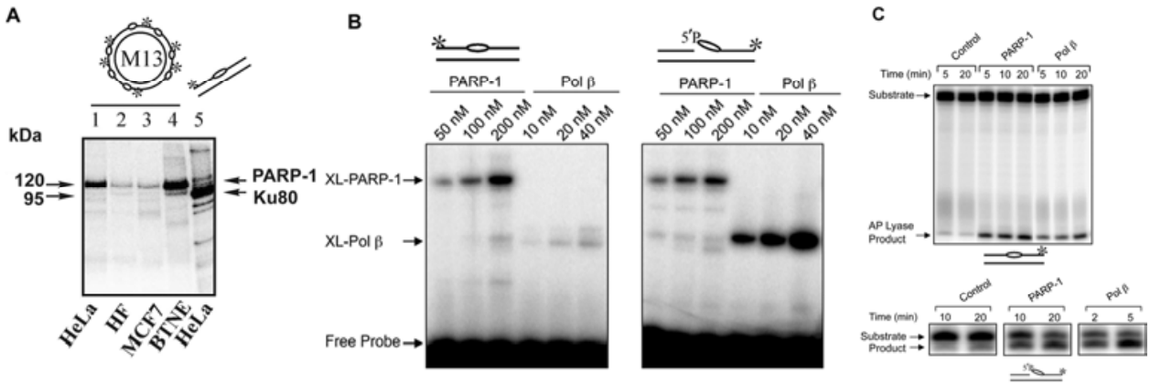

Fig. 4. Interaction of PARP-1 with intact and cleaved AP sites (From Khodyreva et al., 2010b). (A) Cross-linking of mammalian cell extract proteins to circular and linear AP DNA Extracts: whole cell extracts of HeLa, human fibroblasts (HF), MCF-7 and bovine testis nuclear extract (BTNE). (B) Comparison of cross-linking of purified PARP-1 and Pol β with 5'-dRP lyase substrate DNA and AP site-containing DNA. Schematic representations of DNA probes are shown at the top. The * symbol denotes the position of the ^{32}P-label in the DNA. The bubble-like symbol denotes the presence of the AP site in the DNA. (C) 5'-dRP/AP lyase activities of purified PARP-1. The positions of the substrates and products are indicated, and the DNA is illustrated at the bottom.

Cross-linking of PARP-1 was compared with that of Pol β. PARP-1 and Pol β cross-linked to both of these DNA substrates in a concentration-dependent manner. Pol β has a preference for the pre-incised AP site containing-DNA, as compared to the intact AP site. Conversely, PARP-1 does not show a similar preference, yielding similar cross-linking with both probes. The interaction of PARP-1 with the AP site raised the question of the biological relevance of this finding, including whether PARP-1 binds first to the AP site and protects it until repair proteins are recruited. PARP-1 is well known to become activated by binding to DNA strand breaks (Lindahl et al., 1995). Once the AP site became incised by AP endonuclease, PARP-1 became activated and modified by auto-poly(ADP-ribosyl)ation. First, we examined the specificity of PARP-1 interaction with AP site containing-DNA by competition experiments using two types of competitor DNA. A labeled DNA duplex with a 'natural' AP site was used for PARP-1 cross-linking, and the cross-linking was competed either with a control DNA duplex (without an AP site) or a synthetic AP site-containing DNA, tetrahydrofuran (THF), mimicking the AP site. Cross-linking of PARP-1 was reduced with both control and THF-containing DNA. However, the reduction was stronger in the case of

THF-containing DNA than with control DNA. This suggests that PARP-1 has greater affinity for the THF-containing DNA than for the control DNA (data not shown).

The next question regarding PARP-1's interaction with AP sites was whether the enzyme is activated for poly(ADP-ribose) synthesis upon binding to the intact AP site. PARP-1 is well known to become activated by binding to DNA strand breaks (Lindahl et al., 1995), and to avoid the presence of confounding DNA ends, we prepared a double-hairpin DNA for use as probe. First, using this hairpin DNA with internal ^{32}P-label, we confirmed the ability of purified PARP-1 to cross-link to the natural AP site. The results showed that double-hairpin DNA bearing the natural AP site was able to cross-link upon $NaBH_4$ reduction, whereas DNA without the AP site (uracil-DNA) failed to yield cross-linked product (data not shown). As expected, THF-containing DNA failed to cross-link. Next, using similar but unlabeled double-hairpin DNA and ^{32}P-NAD+ as substrate for poly(ADP-ribose) synthesis, we examined the activity of PARP-1. Strong PARP-1 auto-modification was observed only in reaction mixtures containing APE1 (data not shown). PARP-1 auto-modification in reaction mixtures with the natural AP site, but without APE1, was weak; this level, however, was more than the background level (data not shown). Under similar conditions, the THF-containing DNA failed to support poly(ADP-ribose) synthesis, but strong synthesis was observed when APE1 was added. These results indicated that PARP-1 interaction with the intact AP site could result in activation, but this activation involved much less auto-poly(ADP-ribosyl)ation than that observed with APE1-induced strand incision.

Next, to examine PARP-1 auto-modification, purified PARP-1 was first pre-incubated with labeled intact linear AP site-containing DNA. The reaction mixture was then supplemented with NAD^+ to allow poly(ADP-ribose) synthesis. Then, the reaction mixture was treated with $NaHB_4$ and analyzed. The results indicated that poly(ADP-ribose) modified enzyme was cross-linked (data not shown). The mechanism of PARP-1 activation was unclear, but presumably involved single strand break formation within the PARP-1 and DNA complex. In light of this result, we were curious to test PARP-1's capacity to conduct strand incision at the AP site via AP lyase activity. As shown in Fig. 4C, PARP-1 was capable of incising AP site-containing DNA, and the activity was similar to that of Pol β. In light of PARP-1's AP lyase activity, we also tested for 5'dRP lyase activity. PARP-1 conducted 5'-dRP lyase activity against a pre-incised AP site (Fig. 4C), but the activity was much lower than that of Pol β. These results suggested that the endogenous PARP-1 AP lyase activity was sufficient to provide poly(ADP-ribose) synthesis activation at the natural AP site.

Interaction of PARP-1 with AP sites appears to be related with regulation of AP site processing. Such a regulation is particularly important for repair of AP sites included in clustered damage, in which chain breaks, oxidized bases and AP sites are grouped within 1–2 turns of DNA helix and can be situated in both DNA chains. During repair of AP sites within clustered damages additional double strand breaks, which are more cytotoxic, may appear (Yang et al., 2004). PARP-1 influence on hydrolysis of AP sites by APE1 on DNA containing AP site either opposite dAMP or synthetic AP site analogues, was tested (Kutuzov et al., 2011). Along with THF residue, which is most widely used to mimic AP sites, the new AP site analogs were tested (Kutuzov et al., 2011). These analogs were residues of diethylene glycol and decane-1,10-diol. The AP site analogs differ in their sensitivity to the APE1 endonuclease activity. PARP-1 interacts more efficiently with AP sites within clusters that leads to stronger cross-linking with AP sites and more considerable inhibition of APE1 activity as compared with AP DNA containing single AP site.

Thus, by virtue of PARP-1's ability to interact with the intact AP sites (single or within cluster) via Schiff base formation, we demonstrated a new role for PARP-1 in regulation of the BER process. PARP-1's interaction at the AP site could recruit this key enzyme and protect the site until APE1 becomes available to initiate strand incision and BER. Alternatively, PARP-1's 5'-dRP/AP lyase activity could perform strand incision and trigger poly(ADP-ribosyl)ation leading to recruitment of other BER factors.

2.3.4 New function of Neil1 and Neil2 as 5'dRP lyase

According to current view, in mammalian BER the sub-pathway choice is influenced by the rate-limiting step in SN BER, i.e., removal of the 5'-dRP by the dRP lyase activity of Pol β (Horton et al., 2000; Srivastava et al., 1998). For example, if the 5'-dRP cannot be removed efficiently, continued DNA synthesis will emphasize the LP BER sub-pathway (Horton et al., 2000). Yet, both subpathways appear to operate simultaneously to repair most types of DNA lesions *in vitro* (Horton et al., 2000; Prasad et al., 2000). It has been shown previously that the 5'-dRP BER intermediate is the cytotoxic lesion following exposure to methylating agents, and its removal is a pivotal step in BER *in vivo* (Sobol et al., 2000).

Pol β-deficient mouse cells show little dRPase activity (Sobol et al., 2000), but some residual dRP removal by extracts prepared from these cells is still present (Podlutsky et al., 2001). It is possible that while Pol β carries out the bulk of dRP removal from DNA, other activities could be more specifically employed for certain lesions, cell or tissue types, or at certain points of the cell cycle. In *E. coli*, Fpg (formamidopyrimidine-DNA glycosylase) and to a lesser extent endonuclease VIII (Nei) catalyze β-elimination of dRP moiety (Fig. 1). Three mammalian homologues of bacterial Fpg and Nei termed NEIL (Nei-like)-1, -2, and -3 have been identified (Hazra et al., 2002; Hegde et al., 2008). Based on the similarity of their active sites to those of Fpg and Nei, one could expect that they could also display dRPase activity. We have shown that two of these proteins, NEIL1 and NEIL2, are capable of removing dRP lesions from DNA with the efficiency comparable to that of Pol β, and that they can substitute for Pol β dRPase activity in a reconstituted BER assay (Grin et al., 2006).

dRPase activity can be revealed with 3'-labeled nicked abasic oligonucleotide substrates. Such substrates were prepared by end-filling of a 5'-overhanging oligonucleotide duplexes with ^{32}P-labeled dATP and the consecutive treatment of the duplex with uracil DNA glycosylase (Ung) and APE1. The resulting dRP site is unstable in nucleophilic buffers and is degraded during migration through Tris-containing polyacrylamide gels, therefore the products were stabilized by sodium borohydride reduction immediately after the dRPase-catalyzed reaction.

Fig. 5A illustrates that both NEIL1 and NEIL2 possess a dRP-removing activity. The dRPase activities of NEIL1 and NEIL2 demonstrated the enzyme concentration and time dependence expected of an enzyme-catalyzed reaction (Fig. 5B and data not shown). Notably, the activity of NEIL1 in these experiments appeared higher than that of NEIL1 (Fig. 5B). Both NEIL1 and NEIL2 excised with similar efficiency when A, C, or T were placed opposite the lesion, and the excision of dRP opposite G was 1.5–2-fold lower; Pol β removed dRP equally well from all opposite-base contexts. To confirm that dRP removal by NEIL1 and NEIL2 proceeds by β-elimination, as in Pol β and Fpg, we have performed the reaction in the presence of sodium borohydride, which reduces the Schiff base covalent complexes formed between the catalytic amine nucleophile of dRP lyases and C1' of the dRP site during the reaction (Fig. 5C). Such trapped enzyme–DNA complexes are stable enough to be

resolved by regular SDS-PAGE. NEIL1 and NEIL2, as well as Fpg and Pol β, formed low-mobility radioactively labeled complexes.

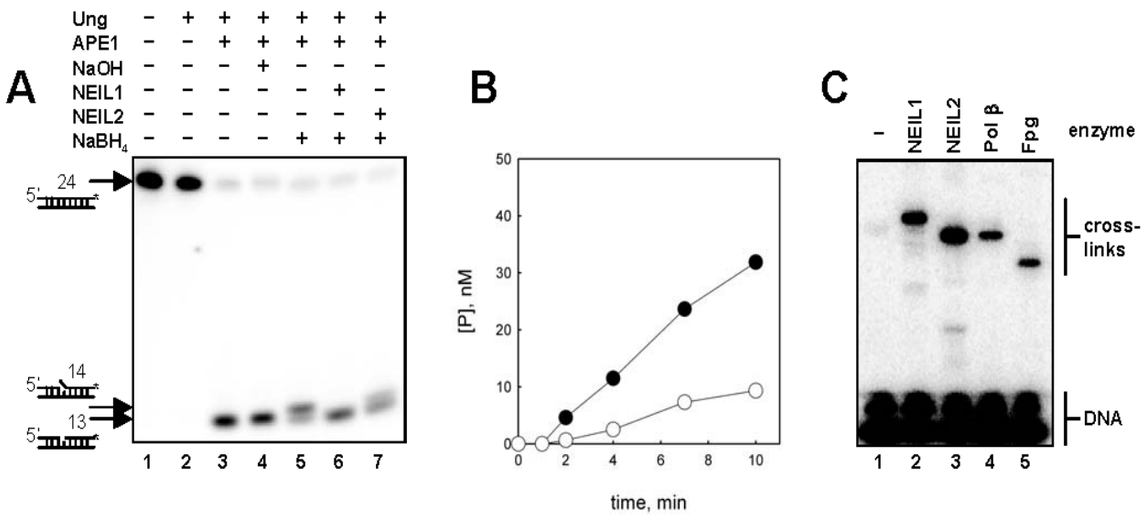

Fig. 5. **dRPase activity of NEIL1 and NEIL2** (From Grin et al., 2006). (A**)** Cleavage of a dRP moiety by NEIL1 and NEIL2. Lane 1, U-containing oligonucleotide; lane 2, AP-containing oligonucleotide; lanes 3–7, dRP-containing oligonucleotide treated with alkali (lane 4), NEIL1 (lane 6) or NEIL2 (lane 7). In lanes 5–7, the dRP-containing oligonucleotide was stabilized with sodium borohydride to prevent its degradation during electrophoresis. Arrows left to the panels indicate positions of the respective oligonucleotide species after PAGE. (B) Time course of dRP excision by NEIL1 (filled circles) and NEIL2 (open circles). (C) Cross-linking of dRP lyases to a dRP-containing substrate by sodium borohydride: lane 1, no enzyme; lane 2, NEIL1; lane 3, NEIL2; lane 4, Pol β; lane 5, Fpg.

To compare the efficiency of NEIL1 and NEIL2 as dRPases with the same activity of DNA polymerase β, the best-known mammalian dRPase, we have determined steady-state enzyme kinetic constants for all three enzymes. The results of these experiments are summarized in Table 1.

	K_M, μM	k_{cat}, min^{-1}	k_{cat}/K_M, $\mu M^{-1} \cdot min^{-1}$
NEIL1	0.21±0.03	0.65±0.04	3.1
NEIL2	2.2±0.7	1.6±0.1	0.74
Pol β	1.0±0.1	3.0±0.1	3.0

Table 1. Kinetic parameters of the dRPase reaction catalyzed by NEIL1, NEIL2, and DNA polymerase β.

The kinetic data suggest that NEIL1 is as good a dRPase as Pol β, and they both surpassed NEIL2 in their ability to remove dRP from DNA. K_M of NEIL1 was ~5-fold lower than K_M of Pol β, indicating that NEIL1 might bind dRP-containing substrate more tightly; on the other hand, Pol β processed the substrate ~5-fold faster than did NEIL1, resulting in nearly equal specificity constants for both enzymes. NEIL2 had an intermediate catalytic constant and the poorest binding of all three dRP lyases compared in this experiment.

The experiments with individual enzymes suggest that NEIL1 and NEIL2 possess dRP lyase activities and could substitute for Pol β in removing dRP moiety in the BER process. To analyze the proficiency of NEIL1 and NEIL2 dRPase in a multienzyme BER process, we have reconstituted the base-excision, AP site-incision, gap-filling and dRP-excision stages of BER using mammalian enzymes (UNG, OGG1, APE1, Pol β (wild type and K35A/K68A/K72A mutant deficient in dRP lyase activity) and NEIL1 or NEIL2. Both NEIL1 and NEIL2 could rescue BER of uracil lesions driven by a dRP-deficient Pol β. The proficiency of NEIL1 in the full BER was higher compared with NEIL2, in agreement with the kinetic parameters showing that NEIL2 is the worst of the three dRPases. We have also reconstituted the repair of AP sites pre-formed in DNA by action of *E. coli* UDG. No major difference from the repair of U was observed.
Having established that NEIL1 and NEIL2 could substitute for dRPlyase activity of Pol β in the reconstituted BER system, we then studied whether NEIL proteins could manifest their dRPase activity in some particular systems, e.g. in cell extracts lacking Pol β.

2.3.5 Identification of HMGB1 as cofactor of the BER process

To identify proteins that have dRP lyase activity or influence removal of the dRP from BER intermediates in the absence of Pol β, we used Pol β null mouse embryonic fibroblast (MEF) cell extract for sodium borohydride driven cross-linking of the Schiff base dRP lyase intermediate protein-DNA complex (Prasad et al., 2007). The strong labeling of a single species in the Pol β null cell extracts corresponding to an unknown protein-DNA complex of 37 kDa was observed (Fig. 6A).
The preferential cross-linking of this protein reflected extraordinary specificity in light of the multitude of proteins in the cell. Taking into account the molecular masses of NEIL1 and NEIL2 (43.5 kDa and 38.2 kDa, respectively) the product could not be related to glycosylases. It should be noted that an apparent molecular mass of a covalent adduct protein-nucleic acid estimated by electrophoretic mobility is approximately equal to the sum of molecular masses of protein and attached nucleic acid.
For identification of protein we applied the approach schematically depicted in Fig. 2. The DNA probe contained a ^{32}P-labeled dRP moiety in a single-stranded break and a 3′-biotin tag to facilitate isolation of cross-linked protein-DNA complexes. Eleven of ions observed in MALDI MS spectrum corresponded to peptides of HMGB1. The $(M + H)^+$ ion of m/z 1520.84 was selected automatically during the data dependent acquisition for MS/MS analysis. The values from both the peptide masses and the MS/MS fragment ion masses were used in a database search. The protein was identified as HMGB1 with a Mowse-based score of 102, 32% sequence coverage and a protein score confidence interval of 99.995%. Among the observed ions the ion of m/z 1520.84 corresponds to amino acid residues 113–127 of the mouse HMGB1 and is a 'signature' that distinguishes HMGB1 from the closely related protein, HMGB2 (Bustin & Reeves, 1996). HMGB1 and HMGB2 are nuclear nonhistone DNA-binding proteins that belong to the high-mobility group box family of proteins (Bustin & Reeves, 1996). HMGB1 has an architectural role in the assembly of nucleoprotein complexes and is highly conserved across species (Bustin & Reeves, 1996; Tang et al., 2010 ;Liu et al., 2010; Stros, 2010). HMGB1 binds to DNA in the minor groove without sequence specificity and has the ability to transiently introduce bends or kinks into linear DNA (Liu et al., 2010; Stros, 2010). The intrinsic ability of HMGB1 to alter DNA structures allows it to participate in many biological processes including regulation of

chromatin structure, transcription, DNA damage repair and recombination. The importance of HMGB1 in DNA repair was identified in studies that revealed the ability of HMGB1 to bind to a variety of bulky DNA lesions (Liu et al., 2010; Stros, 2010).

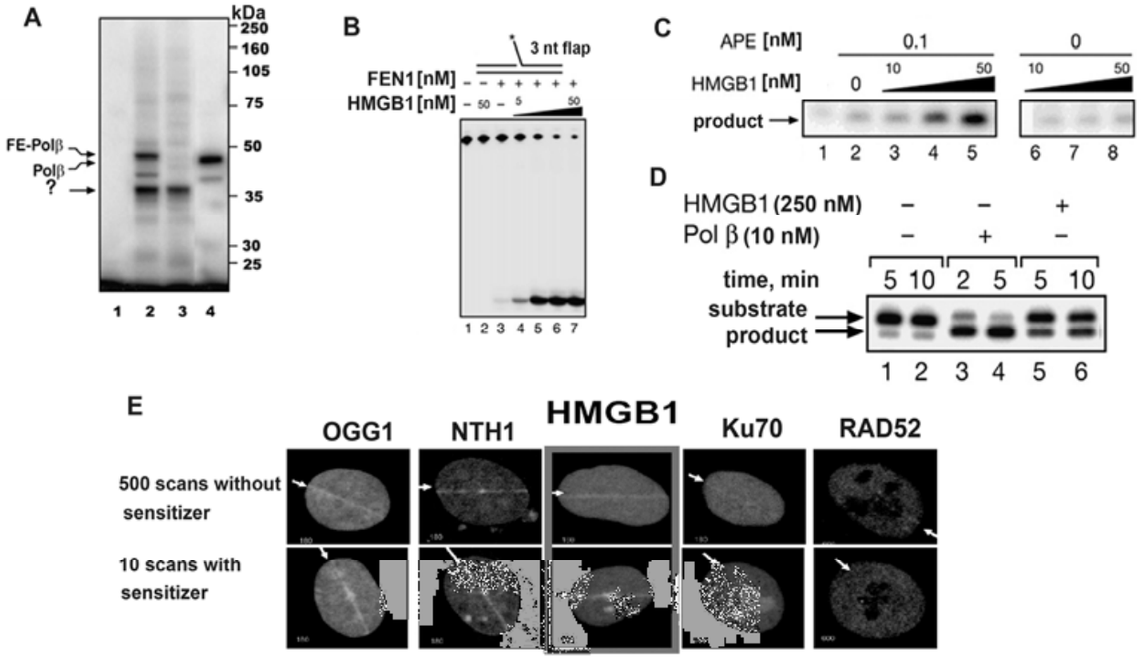

Fig. 6. Identification of HMGB1 as a BER cofactor (from Prasad et al., 2007). (A) Search of extract proteins interacting with the 5'dRP residue in the DNA duplex: lane 2, products of cross-linking between 5' dRP DNA and MEF extract proteins expressing Pol β with flag-epitope (FE), lane 1 control without borohydride treatment. (B) The influence of HMGB1 on FEN1 activity. (C) Influence of HMGB1 on APE1 activity. (D) Comparison of the 5' deoxyribose phosphate lyase activity of HMGB1 and Pol β. (E) Interaction of GFP–HMGB1 in HeLa cells with DNA damage sites induced by scanning laser microirradiation (λ 405 nm) without a sensitizer and in the presence of 8-methoxypsoralen (100 μM). Protein designation: 8-Oxoguanine DNA glycosylase (OGG1); NTH1, DNA glycosylase removing oxidized pyrimidines from DNA; RAD52, protein involved in double-strand break repair, homologous recombination; Ku70, Ku antigen subunit involved in of double-strand break repair, nonhomologous end joining. Arrows show the direction of the scan.

The observed ability of HMGB1 to interact with the BER DNA intermediate poses a question about its role in the process. It was found in the in vitro experiments that HMGB1 isolated from HeLa cells directly interacted with several BER proteins: APE1, Pol β, and FEN1(data not shown) and stimulate the activity of BER enzymes FEN1 and APE1 (Figs. 6B and 6C, respectively). HMGB1 was also revealed to have weak 5' dRP lyase activity (Fig. 6D).
Using HeLa cells expressing HMGB1 in the form of a chimeric protein with green fluorescent protein (GFP–HMGB1), it was found that HMGB1 can be localized in the regions of DNA damage induced by laser microirradiation (Fig. 6E). Irradiation under used conditions generates both single-strand breaks and oxidized bases with high frequency (Lan et al., 2004). Indeed, DNA glycosylases (GFP–OGG1 and GFP–NTH1) efficiently accumulate

in sites of irradiation unlike the proteins recognizing double-strand breaks in DNA (GFP–Ku70 and GFP–RAD52) (Fig. 6E).
Mouse embryonic fibroblasts of the HMGB1+/+ type are more sensitive than HMGB1–/– cells to the combined action of methyl methanesulfonate and methoxyamine, and HMGB1+/+ cells contain a much larger amount of single-strand breaks. The treatment of AP DNA with methoxyamine increases its resistance to APE1 action (Horton et al., 2000).
Another group of researches using two cultivated cell lines of breast cancer found that the increase in the expression level of HMGB1 alters the cells' phenotype by slowing cell growth and increasing the cell sensitivity to ionizing radiation (Jiao et al., 2007).
Interestingly, that in spite of ability of purified NEIL 1/2 to interact with dRP lyase substrate (Grin et al., 2006) we did not reveal abundant products of their cross-linking in the Pol β null MEF extract (Fig. 6 A, lane 2). This interaction appears to be counteracted by effective binding of HMGB1, which is highly abundant in cells.

2.3.6 HMGA as cofactor of the BER process

It is interesting to note that dRP- and AP lyase activities were revealed for another group of chromatin proteins (Summer at al., 2009). Mammalian high mobility group proteins are non-histone chromatin architectural factors encoded by two genes, *HMGA1* and *HMGA2*. Alternative mRNA splicing results in at least four protein isoforms involved in chromatin remodeling and gene transcription (Bustin & Reeves, 1996, Reeves, 2001, Cleynen & van de Ven, 2008). HMGA proteins are characterized by the presence of an acidic C-terminal tail and three DNA binding domains containing short basic repeats, the so called AT-hooks, capable to bind in the minor groove of AT-rich sequences in DNA. In humans, HMGA expression is undetectable or very low in differentiated adult tissues, but high HMGA protein levels are associated with human malignant neoplasias (Berner et al., 1997, Abe et al., 2003, Miyazawa et al., 2004, Meyer et al., 2007). In addition, expression of HMGA1 is functionally linked to chemoresistance of some human carcinomas (Liau & Whang, 2008).
Recombinant human HMGA (HMGA1a, HMGA1b and HMGA2) proteins have been shown to efficiently cleave plasmid DNA containing AP sites (Summer et al., 2009). Further analysis revealed that the proteins could be trapped on AP DNA by $NaCNBH_3$ treatment, the mechanism characteristic of AP lyase activity (David & Williams, 1998; McCullough et al.; 1999, Piersen et al., 2000). To determine the chemical nature of DNA ends generated by the HMGA proteins and the efficiency of AP site cleavage, ^{32}P-labeled double-stranded short DNA duplex containing a single AP site was used as substrate. The analysis revealed that HMGA proteins generated cleavage products, which exhibit the same electrophoretic mobility as those produced by endonuclease III of *E. coli*, an AP lyase catalyzing the β elimination reaction (McCullough et al., 1999; David & Williams, 1998).
To test the possibility that HMGA proteins also possess the related 5′-dRP lyase activity the same DNA duplex bearing label at the 3′ end was employed. A 5′-dRP moiety on the labeled strand was produced by endonuclease IV from *E. coli.* To stabilize chemically labile 5′-dRP group and to improve electrophoretic separation of the products, 5′-deoxyribosyl phosphate moiety was adducted with O-benzyl hydroxylamine. The analysis revealed that the HMGA proteins efficiently removed 5′-dRP moiety. Thus, HMGA proteins display the AP/5′-dRP lyase activity characteristic of the BER process.
Having established that the HMGA proteins are lyases, the authors examined the role of this activity in cell context. To this end, cell lines constitutively expressing HMGA2 have been

generated. Then using transgenic and parental cell lines and employing a variety of cell-based assays and biochemical approaches, the authors provided evidence that the AP site/dRP lyase activities indeed had important biological functions. First, it has been demonstrated that HMGA2 could be efficiently trapped on genomic DNA. Parental cells A549, which express HMGA2 below detectable level, were exposed to low pH or physiological pH as control. DNA isolated from treated cells was incubated with recombinant HMGA2 under trapping (+$NaCNBH_3$) or non-trapping (+NaCl) conditions. The subsequent dot-blot analysis revealed that HMGA2 could be only trapped by DNA derived from cells exposed to low pH, conditions leading to generation of AP sites. Moreover, HMGA2 expressed in transgenic cell line A549 (1.6) was efficiently trapped in a covalent complex *in vivo* with genomic AP sites generated when the cells were subjected to low pH. Analysis of cytotoxic effects that might result from depurination in parental and transgenic cells caused by exposure to low pH revealed that all transgenic cell lines were substantially more resistant than parental cells.

In order to unravel the role of HMGA2 in response of cells to genotoxic impact, parental and transgenic cells were exposed to hydroxyurea (Hu) or methyl methanesulphonate (MMS). Hu is able to induce base oxidation and depurination (Sakano et al., 2001). MMS produces genomic AP sites through the action of DNA glycosylases, which remove the alkylated bases (Sedgwik et al., 2006). In the case of both reagents expression of HMGA2 resulted in significant protection against cell death leading to increase in cell survival.

Both AP and dRP lyase activities play central roles in the early steps of BER (Hegde et al., 2008). In order to demonstrate that protection from MMS induced DNA damage observed with transgenic cells involves HMGA2 lyase activities the cells were sequentially exposed to MMS and O-benzyl hydroxylamine (BA). BA alone had no effect on the survival of parental or transgenic cells. However, combined action of MMS and BA sensitized HMGA2-containg cells to MMS treatment. BA (analogously to methoxy amine) reacts with the baseless deoxyribose moieties (in intact or cleaved AP sites) rendering them refractory to mammalian AP endonuclease 1 and AP/dRP lyase activities (Horton et al., 2000).

Direct interaction of HMGA2 with APE1 *in vitro* and *in vivo* (Sgarra et al., 2008, Summer et al., 2009, Sgarra et al., 2010) has been reported. However, the influence of HMGA2 on the AP endonuclease 1 activity is still unknown. HMGA2 protects cells against three different genotoxicants, i.e. Hu, MMS and low pH (Summer et al., 2009), which introduce the DNA damages repaired by the BER machinery. It is noteworthy here, that repair of these lesions involves the common intermediates, AP sites and 5′ dRP moieties, which can be processed by HMGA2. This strongly support the idea developed in (Summer et al., 2009) that intrinsic AP/dRP lyase activities of HMGA2 are responsible for the protective action of this protein. However, one could not exclude that in addition to direct action, HMGA2 influences the BER capacity indirectly by enhancing the activity of APE1 as was observed in the case of HMGB1 (Prasad et al., 2007). Activation of APE1 by protein-protein interaction may be involved both in the stage of AP site hydrolysis and removing the 3′ end PUA group. APE1 is known as the main mammalian protein capable to excise this blocking group producing the 3′ end hydroxyl moiety (Wilson & Barsky, 2001; Pascucci et al., 2002).

2.3.7 Human ALK B homologue (ABH1) is an AP lyase

Methylated bases in DNA generated by endogenous and environmental alkylating agents can be removed by three distinct strategies. While 3-methyladenine (3-alkyladenine) is

excised by a specific DNA glycosylase that initiates a base excision repair process, 1-methyladenine, 3-methylcytosine and O6-methylguanine are corrected by direct reversal exploring a different mechanism (for more information see a review Sedgwick et al., 2006). One of the strategies of direct reversal involves the activity of DNA dioxygenases, which release the methyl moiety as formaldehyde (Duncan et al., 2002). Although three human DNA dioxygenases – ABH1–ABH3 – catalyze the same oxidative demethylation reaction they display specificity toward methylated base and nucleic acids (DNA or RNA and single- or double-stranded) (Duncan et al., 2002, Westbye et al., 2008, Ougland et al., 2004; Kurowski et al., 2003). Unexpectedly, ABH1 – the closest AlkB *E. coli* homologue – has been shown to display an AP site cleavage activity (Müller et al., 2010).

Intensive study of discovered activity revealed that the DNA cleavage activity of ABH1 did not require added Fe2+ or 2-oxoglutarate, is not inhibited by EDTA, and is unaffected by mutation of the putative metal-binding residues, indicating that this activity arises from an active site distinct from that used for demethylation.

Enzymes that cleave sugar-phosphate backbone at abasic sites can utilize hydrolysis, β- or β,δ-elimination mechanisms (Fig. 1). First, to assess the cleavage mechanism, the activity of ABH1 was examined with DNA containing THF residue, the AP-site analogue, which could not be cleaved by the β-elimination reaction. No AP site cleavage was observed with ABH1 and EndoIII unlike APE1. Second, the electrophoretic mobility of the products resulting from the activities of ABH1, APE1, EndoIII and EndoVIII were examined. Prior to analysis 5′-[^{32}P]-labeled ds-oligonucleotides containing the AP site were incubated with the corresponding enzymes. In some samples, the products of AP site cleavage were additionally treated with T4 polynucleotide kinase (PNK) to remove possible 3′-terminal phosphate by this phosphatase. It should be noted that authors used proteinase treatment of the reaction mixtures to stop the reaction and degrade the enzymes prior to separation of oligonucleotides by denaturing PAAG electrophoresis (Fig. 7). The products produced by ABH1 migrate slowly (Fig. 7, lane 1) than the product of the β-elimination reaction (Fig. 7, lane 3) and do not contain the 3′ end phosphate group since the mobility of the products was not changed by PNK treatment (Fig. 7, compare lanes 1 and 2). While the products derived from the EndoVIII activity, which explores the β,δ-elimination mechanism resulting in the 3′ phosphate group, migrate slowly after PNK treatment (Fig. 7, compare lanes 5 and 6). The authors proposed that ABH1 cleaves AP sites by β-elimination with ABH1 being bound with the product in tight complex. They attribute the slight decrease in mobility of the products for the ABH1 samples to tight binding of ABH1 fragments with oligonucleotides. The authors demonstrated that both ABH1 and EndoIII in the presence of $NaBH_4$ are able to generate stable products with single-stranded AP-DNA, double-stranded DNA containing one AP site and double-stranded DNA containing two AP sites, but the important control without reducing agent is missed. Taking into account that ABH1 forms stable adducts with AP DNA without reduction, as observed in the activity test (Fig. 7, lanes 1 and 2), it is questionable whether the trapping of ABH1 is the Schiff base dependent.

ABH1 was shown to display specificity in substrate usage with DNA containing two AP sites being the preferable substrate. Further analysis of AP site cleavage activity at different substrate-to-enzyme ratio demonstrated that concentration of product was always sub-stoichiometric to the enzyme concentration that is in agreement with tight binding of ABH1 with the product.

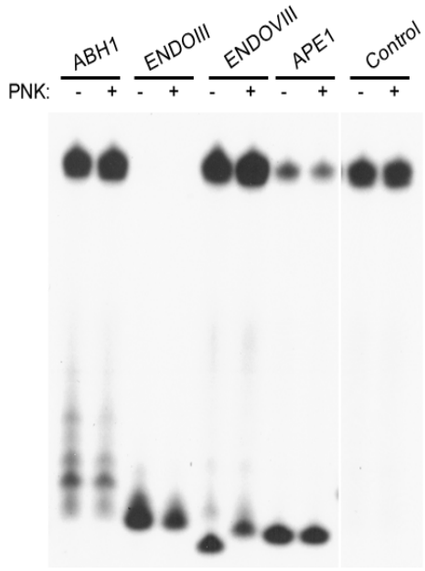

Fig. 7. Comparison of product sizes for ABH1, APE1, ENDOIII, and ENDOVIII, and examination of the effect of phosphatase treatment (from Müller et al., 2010). The uracil containing DNA duplex was cleaved by incubation with ABH1 or the control endonucleases in the presence of UDG for 1 h at 37 ºC. Portions of the samples were treated with the phosphatase T4 polynucleotide kinase to remove possible phosphates at the 3′ end of the labeled products. The presence of a 3′-phosphate causes the oligonucleotide to migrate more rapidly than the non-phosphorylated species due to the extra negative charge; removal of the phosphate results in a shift to an apparently larger product, as seen with EndoVIII.

Taking into account ubiquitous expression of APE1 and inefficiency of the ABH1 AP lyase activity, ABH1 hardly play significant role *in vivo* in processing of AP sites. Moreover, the ability of ABH1 to more efficiently cleave opposite AP sites in ds DNA that may result in formation of DS breaks, more toxic for the cells than AP sites, therefore action of ABH1 on clustered AP sites in genomic DNA appears to be dangerous for cells. The authors considered tight binding of ABH1 with the product as a mechanism that protects ends from degradation. On the other hand, tight binding may create hindrances for the repair processes and require special efforts to remove blocking group from the 3′ end. Potentially interesting finding of intrinsic AP lyase activity of ABH1 requires additional studies to draw a conclusion concerning significance of discovered activity *in vivo*.

2.3.8 Tyrosyl-DNA-phosphodiesterase mediates the new APE-independent BER pathway in mammals

As mentioned above AP sites can be cleaved by activity of bifunctional DNA glycosylases with associated AP lyase activities via β- or β,δ-elimination mechanism producing DNA intermediates with 3′ end containing 3′-phosphate or 3′-PUA groups (Fig. 1) that have to be removed prior to DNA synthesis may occur. DNA intermediates with blocked 3′end may also appear from action of ROS and as a result of spontaneous decomposition of AP sites.
The 3' PUA is known to be removed by the only AP-endonuclease, APE1, which possesses 3′ phosphodiesterase activity with α-unsaturated aldehydes, producing a single nucleotide gap flanked by a 3′-hydroxyl group and a 5′ phosphate group (Wilson & Barsky, 2001;

Pascucci et al., 2002). However, APE1 is barely active in removing 3' phosphate generated by mammalian DNA glycosylases NEIL1 and NEIL2. The 3' phosphate is efficiently removed by polynucleotide kinase (PNK) and not APE1 (Wiederhold et al., 2004).

Tyrosyl-DNA phosphodiesterase (Tdp1) was discovered as an enzymatic activity from *Saccharomyces cerevisiae* that specifically hydrolyzes the phosphodiester linkage between the O-4 atom of a tyrosine and a DNA 3′ phosphate (Yang et al., 1996). This type of linkage is typical for the covalent reaction intermediate produced upon Topoisomerase 1 cleavage of one DNA strand. Human Tdp1 can also hydrolyze other 3′-end DNA alterations that are covalently linked to the DNA, indicating that it may function as a general 3′-DNA phosphodiesterase and repair enzyme (Dexheimer et al., 2008). Tdp1 can also remove the tetrahydrofuran moiety from the 3'-end of DNA (Interthal et al., 2005). It is conceivable that Tdp1 acts on the 3′ PUA moiety.

To study an ability of Tdp1 to process the 3′ PUA moiety AP DNA was first incubated with Endo III (Fig. 8A, lane 2) (Lebedeva et al., 2011). Following incubation of this product with Tdp1 results in 15-mer with 3' phosphate, which can be removed by PNKP (Fig. 8A, lane 3). Thus, Tdp1 is able to remove the 3′ PUA that allows to realize the APE1-independent pathway of BER where AP sites are cleaved by bifunctional DNA glycosylases via the β-elimination mechanism.

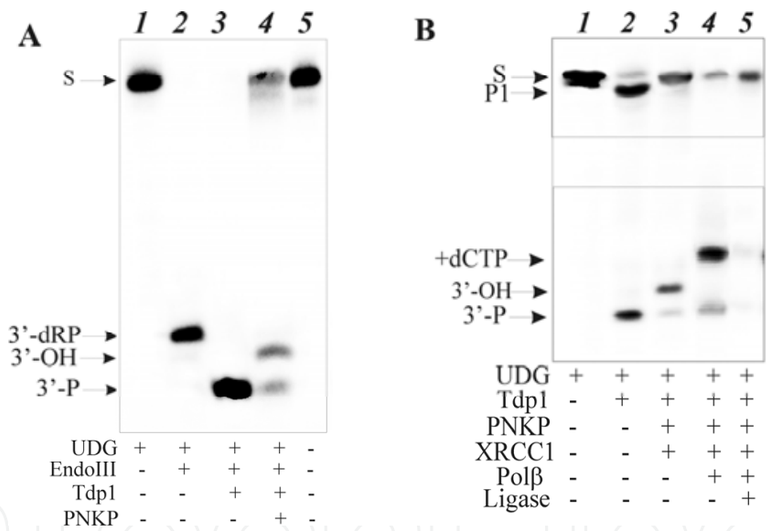

Fig. 8. Tdp1 activity on DNA substrate with 3'-dRP moiety in the single strand break (A) and reconstitution of the AP-DNA substrate repair initiated by Tdp1 (B) (From Lebedeva et al., 2011)**. (A)** The 15-mer with 3'-dRP was generated by incubation of AP DNA with EndoIII (lane 2). Following incubation of this product with Tdp1 results in 15-mer with 3'-P (lane 3). Lane 4 shows the 15-mer product with 3'-OH after adding PNKP in the reaction mixture. Lane 1 corresponds to the AP-DNA substrate incubated with UDG. Lane 5 - control. (B) 5'-end labeled AP-DNA substrate was subsequently incubated with the UDG (lane 1), Tdp1 (lane 2), PNKP and XRCC1 (lane 3), Pol β (lane 4), DNA ligase III (lane 5). The components present in different reaction mixtures are indicated.

We tested an ability of Tdp1 to process AP sites (natural or mimicked by THF residue) and found that Tdp1 cleaves both types of AP sites generating the product identical to the product of β,δ-elimination (data not shown). Finally, the repair of AP site was analyzed in a

minimal reconstituted BER system consisting of purified proteins (Fig. 8B). The 5′ ^{32}P-labeled 32-mer DNA duplex containing uridine at the position 16 was incubated with the purified recombinant UDG, Tdp1, PNKP, Pol β, DNA ligase III, and XRCC1 to mimic DNA repair system. The reaction mixture containing Tdp1 but lacking PNKP (lane 2) generated a product with a 3′-phosphate, which is identical to that produced by NEIL1. Addition of PNKP resulted in a 15-mer product with the 3′-OH termini (lane 3). XRCC1, a scaffold protein, stimulates the activity of BER proteins, such as DNA polymerase β, PNKP, DNA ligase III (Mani et al., 2004; Caldecott, 2003; Whitehouse et al., 2001).
Lastly, DNA polymerase β replaces the missing DNA segment (lane 4) and DNA ligase reseals the DNA (lane 5). So, the repair of AP site initiated by Tdp1 fully restored the intact DNA and generated the products of the expected lengths at each intermediate stage. In summary, human Tdp1 protein can initiate APE1-independent repair of AP sites and 3′ PUA termini that expands the ability of the BER process.

2.3.9 XRCC1 interactions with base excision repair DNA intermediates

XRCC1 is known to play a crucial role in the coordination of two overlapping repair pathways, SSBR and BER (Caldecott, 2003). Although the main role of XRCC1 during BER has been attributed to its participation in the post-incision steps (Wong et al., 2005), which are shared with SSBR, the physical and functional interactions with proteins involved in the initiation of modified bases or abasic sites repair (Marsin et al., 2003, Campalans et al., 2005, Vidal et al., 2001, Wiederhold et al., 2004) suggest that XRCC1 presence at the early steps of BER could be important for assuring a correct repair process. One possible role of XRCC1 could be to optimize the passage of DNA substrates from one enzyme to the next one in the pathway by holding the proteins together through its different interacting domains (Caldecott, 2003).
Investigating the interactions of with different BER DNA intermediates generated either by DNA glycosylase hOGG1 or AP endonuclease APE1 we have found that XRCC1 is able to interact with AP sites via formation of the Schiff base intermediate (Nazarkina et al., 2007). Because hOGG1 possesses both, DNA glycosylase and AP lyase activities, either of two DNA intermediates can be produced: DNA duplex containing an intact AP site or a nick with a 3′ PUA moiety (See Fig. 1). By competition experiments using the THF-containing or regular DNA duplex it was demonstrated that XRCC1 binds DNA with an AP site, or its synthetic analogue, with considerably higher affinity than regular DNA duplex.
XRCC1 is known to bind DNA with single-strand breaks with higher affinity that regular DNA duplex (Mani et al., 2004). We then investigated the relative affinities of XRCC1 to different DNA structures that could be BER intermediates in the repair of single or double DNA lesions. The efficiency of cross-linking is maximal with an incised AP site 3′ PUA (Fig. 9A, lane 3). Interestingly, the presence of a strand interruption on the complementary oligonucleotide strongly stimulated the cross-linking of XRCC1 to the AP site. These results suggest that XRCC1 could be important to hold the DNA together during the repair of clustered DNA damage. XRCC1 is also able to cross-link to a 5′ dRP residue downstream of a nick (Fig. 9A, lane 6). Comparative analysis of the patterns of protein cross-linking to AP DNA in cell extracts deficient (EM9) and proficient in XRCC1 (EM9-X) (Fig. 9B, lanes 3–6) revealed the product that can be related with XRCC1 (lane 6). Interestingly, that the band is observed with DNA containing interruption opposite AP site (Fig. 9B, lanes 4 and 6).

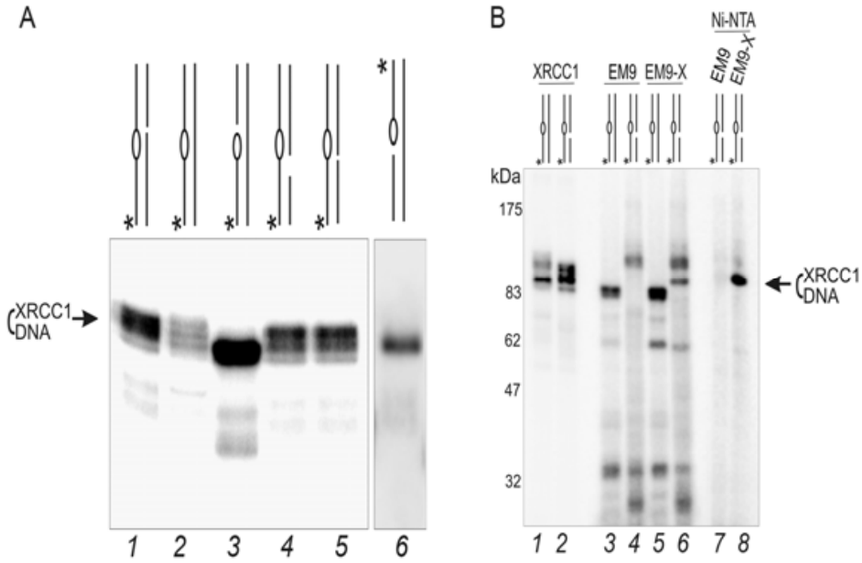

Fig. 9. Interactions of XRCC1 with BER DNA intermediates (From Nazarkina et al., 2007). (A) Cross-linking of XRCC1 to different 5′-^{32}P-labeled AP DNA. DNA containing intact AP sites (lanes 1, 2, 4, and 5) and cleaved AP site (3′ PUA) (lane 3) were 5′ end labeled, while DNA containing hydrolyzed AP site (5′ dRP) (lane 6) was 3′ end labeled. (B) Cross-linking of XRCC1 with AP DNA in CHO cell extracts deficient in XRCC1 (EM9) or EM9 expressing His-tagged human XRCC1 (EM9-9). Position of radioactive label in DNA is designated by the asterisk.

To confirm that this product corresponds to the XRCC1 containing conjugate, after trapping with $NaBH_4$, His-tagged XRCC1 was recovered by pull-down using a Ni-NTA resin. After this purification step, the product was recovered from the EM9-X extract (lane 8) but not the EM9 one (lane 7).

Taken together, these results demonstrate XRCC1 ability to interact with intact and cleaved AP sites, including cell extracts, e.g. in the presence of cellular proteins that can interfere with XRCC1 binding to AP DNA.

Thus, using the Schiff-base-mediated cross-linking, we show that XRCC1 displays a specific affinity for AP containing substrates. Although at this time we cannot evaluate the in vivo relevance of covalent complexes between XRCC1 and DNA, considering the Schiff base reversibility, it is tempting to speculate that its formation during BER of AP sites could be a physiological response to situations where a reactive intermediate needs to be protected until the next enzyme recruited by XRCC1 is able to process it.

3. Conclusion

Combination of affinity based cross-linking of proteins with specific DNA probes containing intact or cleaved AP sites and MS analysis allowed to identify new players in recognition/processing of these ubiquitous lesions. Some identified proteins are known as regulatory proteins of specific DNA repair processes, not necessarily involved in repair of AP sites, but all these proteins are related to cell radiosensitivity. Two of them—XRCC1 and PARP1—belong to the base excision repair system, but have been previously considered as participants of later stages of the BER process. By virtue of its interaction with AP sites,

PARP1 and XRCC1 can regulate initial stage of BER persisting at AP sites until APE1 could come and initiate AP sites cleavage. The PARP1 activation upon interaction with AP sites and resultant automodification appear to facilitate the BER factor recruitment to stimulate the repair process. In the absence or deficiency of APE1, PARP1 can provide temporal protection of AP sites. Ku antigen can temporarily protect AP sites situated within 1-2 turns of DNA helix from double-strand breaks or displays AP/dRP lyase activity near double-strand ends. The last activity has no analogues and is indispensable for proper repair of DS breaks with particular blocking groups at the ends, which can result from action of ionizing radiation.

In other case of revealed AP/dRP lyase activities, they appear to function under particular physiologic or stressful conditions modulating the capacity of the BER system or providing the appropriate functions of the repair machinery when some participants are missed or inactive. Interestingly, that structural chromatin proteins –HMGB1 and HMGA – were found to interact with AP sites displaying an enzymatic activity. Although being a relative weak, this activity may be biologically significant due to high abundance of these proteins.

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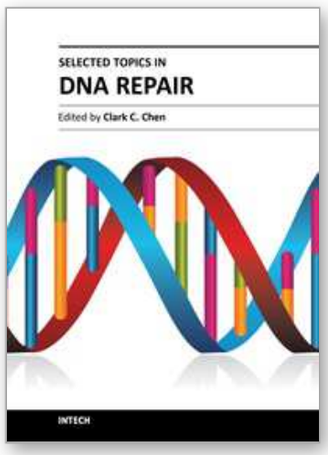

Selected Topics in DNA Repair

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This book is intended for students and scientists working in the field of DNA repair, focusing on a number of topics ranging from DNA damaging agents and mechanistic insights to methods in DNA repair and insights into therapeutic strategies. These topics demonstrate how scientific ideas are developed, tested, dialogued, and matured as it is meant to discuss key concepts in DNA repair. The book should serve as a supplementary text in courses and seminars as well as a general reference for biologists with an interest in DNA repair.

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