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Ciclo XXX

Titolo della tesi

"Novel roles of DNA damage repair enzymes in the processing of modified ribonucleotides embedded in DNA"

Dottoranda

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To my father ...

because "happiness can be found even in the darkest of times ...

... if one only remembers to turn on the light"

Summary

1. Preface

The erroneous incorporation of ribonucleotides into genomic DNA occurs very frequently in cells during physiological processes. Several works published in literature have demonstrated that more than 100 million rNMPs are transiently present in mammalian DNA during each replication cycle, as a consequence of: *i)* the inability of DNA polymerases to discriminate rNTPs from dNTPs; *ii)* the abundance of rNTPs compared to dNTPs in the nucleotide cell pool and *iii)* the presence of residual ribonucleotides belonging to RNA primers derived from the incomplete repair of the Okazaki fragments.

The presence of RNA, as single or more ribonucleotides, into DNA results very dangerous for the cell due to its distorting effects on the double helix of DNA. For this reason, a specific pathway acts in order to remove this lesion called Ribonucleotide Excision Repair (RER) pathway, in which RNase H2 plays an important role as endonuclease, able to cleave at the 5' side of normal rNMPs in DNA. Although in the last decade huge steps forward have been done in this field, more studies are needed for better understanding the impact of this lesion on DNA and their back-up repair mechanism when the RER mechanism does not work, as it happens in several pathologies including cancer and Aicardi-Goutieres syndrome.

Notably, nothing is known about the putative ability of this pathway to repair modified (i.e. oxidized or abasic) rNMPs within DNA.

Moreover, because of the importance of the Base Excision Repair (BER) in repairing non-bulky lesions, including oxidized and abasic bases, a role of the BER mechanism in the removal of rNMPs embedded in DNA needs to be addressed.

The Apurinic/apyrimidinic endonuclease 1 (APE1) is the main protein that works in BER pathway as abasic endonuclease. Deoxy- abasic sites, generated spontaneously or following the previous processing by glycosylases on a damaged (including oxidized) base, are efficiently cut by APE1. The importance of APE1 in the BER pathway also comes by its altered expression observed in different human pathologies including neurodegenerative disorders and cancer. Moreover, APE1 has nucleotide incision repair (NIR)

activity on different modified bases, such as 5,6-dihydro-2'-deoxyuridine, 5,6-dihydrothymidine, 5-hydroxy-2'-deoxyuridine, 5-hydroxycytosine, which are directly repaired by APE1 bypassing the action of specific glycosylases. Last, but not least, the role of APE1 as a redox co-activator of several transcription factors by modulating their DNA binding ability is well ascertained.

Findings from our and other laboratories revealed an interesting involvement of BER in RNA metabolism. Specifically, APE1 can endonucleolytically cleave abasic single-stranded RNA, has a 3′-RNA phosphatase activity, and a weak 3′-5′ exoribonuclease activity.

Upon demonstration of the involvement of the BER mechanism in RNA metabolism, we hypothesized that BER enzymes could be involved in the processing of rNMPs in DNA, particularly in the case of chemically modified rNMPs, such as abasic and oxidized rNMPs.

Although published data have demonstrated that spontaneous depurination occurs ~1,000 times slower in RNA than DNA, due to the high abundance of rNMPs in genomes, with more than 100 million rNMPs transiently present in mammalian DNA, the possibility that abasic and oxidized rNMPs (such as 7, 8-Dihydro-8-oxo-riboguanosine) are present in DNA and could be considered targets of BER is quite real. These data, together with other recent findings of the ability of *S. pombe* Pol 4, *M. smegmatis* DinB2 and human Polβ to insert and elongate oxidized rGMP, underscore the necessity to determine how cells can target and remove oxidized or abasic rNMPs from DNA.

Identifying and discovering whether BER may target normal and modified rNMPs in DNA, can also help to better understand the mechanism of genotoxicity of reactive oxygen species as well as the function and the impact of BER defects in human disease and cancer mechanisms.

For all these above reasons, the main focus of this Thesis is to test the hypothesis of whether BER enzymes may process oxidized or abasic rNMP incorporated in DNA.

2. Abbreviations

- DDR: DNA Damage Response
- ROS: Reactive Oxygen Species
- SSB: Single Strand Break
- d8oxoG: 8-oxo-7,8-dihydroguanine deoxyribose
- AP: Abasic site
- BER: Base Excision Repair
- OGG1: 8-oxoguanine DNA glycosylase
- APE1: AP-endonuclease 1
- NIR: Nucleotide Incision Repair
- dNMPs: deoxyribonucleotides monophosphate
- rNMPs: ribonucleotides monophosphate
- RER: Ribonucleotide Excision Repair
- RNase H: Ribonuclease H
- AGS: Aicardi Goutières Syndrome
- rAP: Ribo- abasic site
- r8oxoG: 8-oxo-7,8-dihydroguanine ribose

3. Figure *Index*

4. Introduction

4.1 DNA damage *versus* DNA repair: an overview

During its life-span, eukaryotic cells are continuously exposed to innumerable environmental conditions, including smog, infectious agents, tobacco and UV light, defined as damaging agents because having the ability to injury cellular macromolecules (e.g. DNA, lipids and proteins) (Hoeijmakers 2001).

DNA is an intrinsically reactive molecule whose integrity should be preserved as long as possible in order to guarantee genomic inheritance. If DNA is damaged, the consequences for the cell and, consequently, for the whole organism, could be disastrous (Chan and Dedon 2010).

Among DNA damaging agents, a subdivision in two categories based on the origin of the damaging agents is preferred:

- *endogenous agents,* in which damages induced by physiological cellular processes are included, such as: replication errors, oxygen metabolism, DNA base mismatches by polymerases, the formation of topoisomerase-DNA complexes, spontaneous DNA deamination, methylation and abasic sites, ribonucleotides incorporation;
- *exogenous agents,* in which chemical and physical agents are considered and which are provoked by the environment such as: ionizing radiation, UV radiation, alkylating agents, aromatic amines, toxins.

Replication errors, DNA base mismatches, base deamination / alkylation / methylation, generation of oxidized and abasic sites are some of several DNA injuries (Figure 1). If unrepaired, DNA damage can be the cause of cell death or aberrant cell proliferation contributing to premature aging and cancer (Wilson and Bohr 2007). To avoid this, each cell is well-equipped with genome maintenance systems including DNA repair, cell cycle checkpoint and cell death pathways that ensure keeping the DNA integrity (Bauer, Corbett, and Doetsch 2015).

DNA **D**amage **R**esponse (DDR) pathways include several DNA repair systems that work in a sophisticated way to recognize and repair a specific

type of DNA damage and re-establish the genome integrity. Among the several active DDR mechanisms, there are: the Base Excision Repair, Nucleotide Excision Repair, Mismatch Repair, Homologous Recombination and Non-homologous End Joining pathways (Figure 1) (Chatterjee and Walker 2017).

Although each DNA repair pathway has been originally characterized as single entities acting on a specific subtype of DNA lesion, a crosstalk between several DNA repair pathways operates in order to properly repair the DNA injury.

An imbalance between DNA damage and repair, or a disruption of DDR systems or damage tolerance, increases the risk of genomic instability and mutagenesis, promoting several diseases, including cancer, aging or neurodegenerative disturbs (Hoeijmakers 2009).

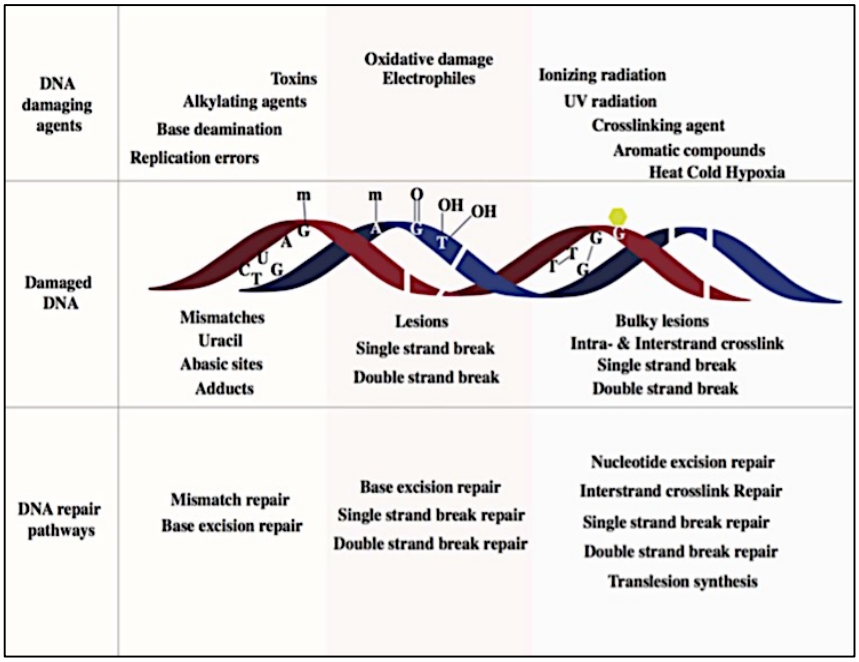


Figure 1 ***Overview of different DNA damaging agents, DNA effects, and relative DNA repair pathways***

The figure, divided into three panels, recapitulates for each DNA damaging agent (upper panel), the lesion induced on DNA (Damaged DNA - middle panel) and finally DNA repair pathways acting on the lesion (lower panel). (Chatterjee and Walker, 2017)

In next paragraphs, our attention will be focused on two important DNA lesions induced by oxidative stress and rNMP incorporation and their relative damage response responsible for restoring the DNA stability.

4.2 Oxidative stress and its impact on DNA

Eukaryotic organisms activate a process called *aerobic respiration* within mitochondria in which several mitochondrial enzymes work to promote the conversion of glucose in oxygen to produce CO_2, water and energy.

During the one-electron reduction of oxygen of the aerobic respiration, several oxidizing species are generated including superoxide radical ($O_2^{\bullet-}$), non-radical hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). Some of them are defined as **R**eactive **O**xygen **S**pecies (ROS), highly reactive product of oxygen metabolism (Gebicki 2016). ROS produced during oxidative stress are considered as the most dangerous trigger of damage for several macromolecules including proteins, lipids and, overall, nucleic acids (Balazy and Nigam 2003). In fact, they are electron deficient, so they readily oxidize cellular macromolecules particularly DNA and RNA.

The first product generated during oxygen metabolism is the superoxide anion radical ($O_2^{\bullet-}$). 1-1.5% of the oxygen intake results in the production of $O_2^{\bullet-}$. $O_2^{\bullet-}$ is converted to hydrogen peroxide (H_2O_2) through an enzymatic dismutation process (Boveris and Cadenas 2000; Valdez et al. 2000). Both $O_2^{\bullet-}$ and H_2O_2 are low reactive against biomolecules, so they are not considered highly dangerous for the cell. When H_2O_2 gets in contact with transition metals, such as ferrous ions, it is reduced through a Fenton reaction generating hydroxyl radicals ($^{\bullet}OH$).

Contrarily to their precursors, $^{\bullet}OH$ radicals are highly reactive products that are the direct cause of oxidative damage to cellular macromolecules (Cadet et al. 2017; Kalyanaraman 2013).

Moreover, during pathological situations, including type 2 diabetes, atherosclerosis and cancer, generation of OH products is increased, and is often coupled to the activation of NO-synthase that triggers the release of nitric oxide ($^{\bullet}NO$) (Boveris et al. 2000). When $^{\bullet}NO$ reacts with $^{\bullet}OH$, the combined peroxynitrate $ONOO^-$, a powerful reactive nitrogen species (RNS), is produced resulting dangerous for the cell (Bustamante et al. 2000; Cadenas et al. 2000).

Not only aerobic respiration is the cause of ROS generation. In fact, oxidative stress can also be induced by exposure to diverse chemical and

physical agents such as ionizing radiations, UV radiation, redox cycling drugs, carcinogenic compounds and toxins which are critical fonts of damage induced by the formation of singlet oxygen ($^1O_2$) or OH radicals (Radman 2016).

All purine and pyrimidines can be attacked upon oxidative stress (Miral Dizdaroglu 2012) in both cellular nucleotide pool or directly into DNA (Figure 2). A multiplicity of structurally different lesions are formed upon endogenous and exogenous oxidation. Among all the five bases, the guanine base is the more susceptible base to oxidative stress because it possesses the lowest redox potential (Cadet 2014). The most important oxidative conversion of the guanine is into 8-oxo-7,8-dihydroguanine (8oxoG). 8-oxoguanine can originate as 8-oxo-dGTP, generated in the nucleotide pool or by direct oxidation of the DNA guanine base. This modified base is so abundant within the cell that it is usually used as a cellular biomarker for oxidative stress (Collins et al. 1996). 8oxoG accumulates in both nuclear and mitochondrial DNA and, because of its frequency, it is believed to be the major cause of cancer (Nakabeppu 2014). Moreover, reduction of the guanine can lead to the generation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG). Both FapyG and 8oxoG are efficiently removed by eukaryotic OGG1, that will be deeper explained in the next paragraph (David, O'Shea, and Kundu 2007).
As for guanine, also adenine can be attacked by ROS giving rise to 8-oxo-7,8-dihydroadenine (8-oxoA), 4,6-diamino-5-formamidopyrimidine (FapyA) and 2-hydroxyadenine. Thymine glycol and cytosine glycol are the most common products following oxidative stress of thymine and cytosine respectively (Figure 2) (Breen and Murphy 1995; Dizdaroglu et al. 1986).

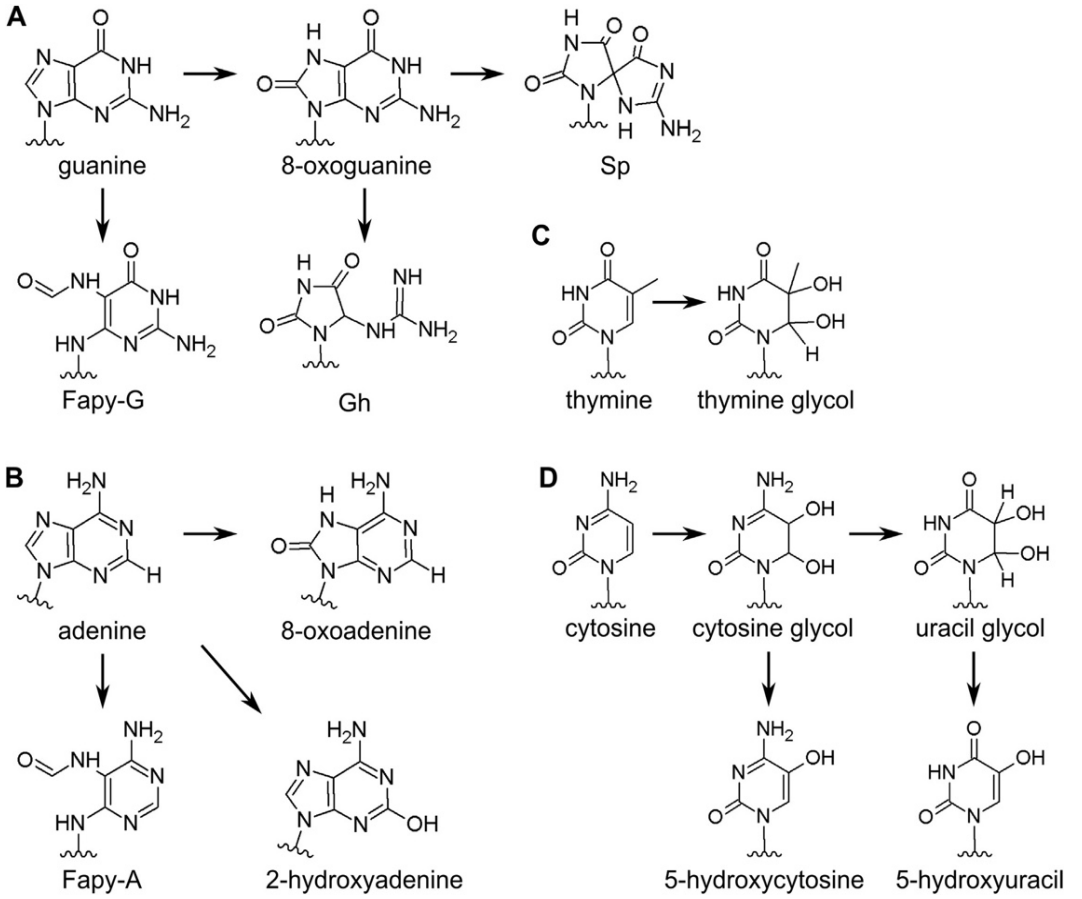


Figure 2 ***Structure representation about most frequent modified bases that commonly occur upon oxidative stress***

All purine and pyrimidines are subject to modification upon oxidative stress. (Lee and Wallace, 2016)

8oxoG can also be further oxidized to two major products, spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) (Figure 2) (Neeley and Essigmann 2006). Sp and Gh are removed by NEIL1 and NEIL2 glycosylases, through β,δ-elimination mechanism in double and single strands respectively. NEIL3 also acts on these substrates and in oxidized pyrimidines (Krishnamurthy et al. 2008).

Oxidized dGMP assumes an important role in genomic instability when embedded in DNA. If not readily repaired, it can cause a G→T transversion mutation. Upon succeeding DNA replications, 8oxoG will pair with adenine on the Hoogsteen face rather than cytosine on the Watson Creek face fixing a T mutation in the new DNA strand (Figure 3). This mutagenic state can activate a carcinogen process (Lonkar and Dedon 2011). On the contrary, when 8oxoG

or FapyG are paired with adenine, eukaryotic MUTYH, a MMR enzyme, will preferentially remove adenine in the opposite strand (Takao et al. 1999) giving another opportunity to the polymerase to incorporate a correct cytosine (Figure 3).

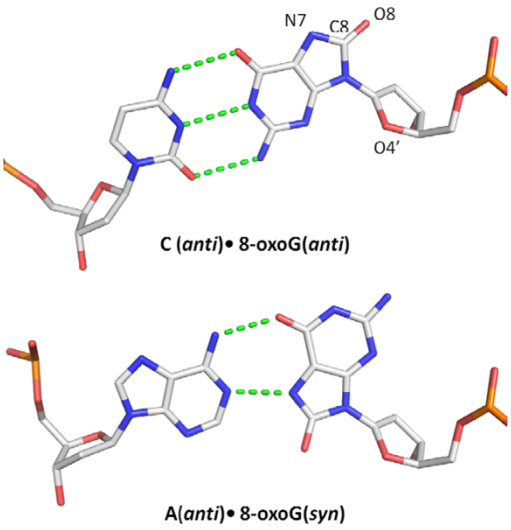


Figure 3 ***Two matches are possible when G base is oxidized***

8oxoG can pair with cytosine following Watson Creek face (upper) or with adenine following Hoogsteen face (bottom). In the last case, a mutation will be generated under subsequent replications. (Faucher et al., 2012)

When lesions induced by oxidative stress are not efficiently repaired, the effects on the organism are different, including: aging, cancer and neurodegenerative disorders (Islam 2017). Recently, a new unexpected concept has been hypothesized. Data obtained *in vitro* show that the presence of 8oxoG containing in transcription factor binding sequences modulates negatively the binding affinity of the transcription factor to the DNA. This effect was demonstrated in several cases including in SP1, NFkB and CREB sequences, some of the most important transcription factors (Fleming, Ding, and Burrows 2017; Fleming and Burrows 2017). Actually, it appears that the presence of d8oxoGMPs in DNA may epigenetically impact on gene transcription, thus opening new questions on non-canonical roles of DNA lesions and their impact *in vivo*.

In the next paragraph, the pathway deputed on repairing lesions induced by oxidative stress is described.

4.3 Base Excision Repair pathway

BER is a ubiquitous pathway, considered one of the most important DNA repair pathway for maintaining genomic integrity to oxidative-based damage on DNA (Markkanen 2017). The importance of the BER pathway is easily recognized in those cases in which genes of the core BER factors are deleted, resulting in embryonic or early post-natal lethality (Iyama and Wilson 2013).

The BER mechanism is able to repair DNA damage that does not induce DNA helix distortions called non-bulky lesions. These lesions can be generated by several DNA damaging agents, including: alkylating, deaminating and oxidative agents, both in nuclear and mitochondrial DNA (Dianov et al. 2001; Krokan and Bjoras 2013).

Briefly, the BER pathway is composed of five essential steps, which are well conserved from Bacteria to Eukaria (Figure 4):

i) The first action is carried on by lesion-specific DNA glycosylases. These enzymes recognize the damaged base through a flipping out mechanism and cleave it through a sophisticated process (Wallace 2013).

ii) After the hydrolysis of the *N*-glycosyl bond by DNA glycosylases, abasic (AP) sites are generated. AP sites are processed by a specific apurinic/apyrimidinic endonuclease, APE1 (Bauer, Corbett, and Doetsch 2015). APE1 cleaves the phosphodiester bond generating two free termini, 3' OH and 5' phosphate.

iii) The abasic dRP intermediate generated is then removed by Polβ which also inserts the correct nucleotide (Howard and Wilson 2017).

iv) Finally, the ligation of the residual nick is carried on by Ligase III (Howes and Tomkinson 2012).

It has been estimated that up to 10,000 abasic sites are formed per human genome *per* day by spontaneous hydrolysis of the *N*-glycosyl bond (Dianov and Hübscher 2013; Lindahl 1993), thus the BER mechanism can work independently of DNA glycosylases when an AP site is generated.

An important diversification of the BER pathway is based on the step carried on by the polymerase. The pathway can be completed via a “short patch” (SP) or a “long patch” (LP) mechanism.

In the SP pathway, Polymerase β is engaged to replace the single missing nucleotide, then Ligase I or a XRCC1-Ligase III complex is responsible for the ligation of the nick (Sobol et al. 1996). In the LP via, preferentially chosen during the S-phase of the cell cycle, FEN 1 (flap endonuclease 1) excises the displaced strand while Polymerases δ and ε work with the sliding clamp PCNA (proliferating cell nuclear antigen), generating an 2-12 nucleotides strand. The nick is finally sealed by Ligase I (Sung, DeMott, and Demple 2005).

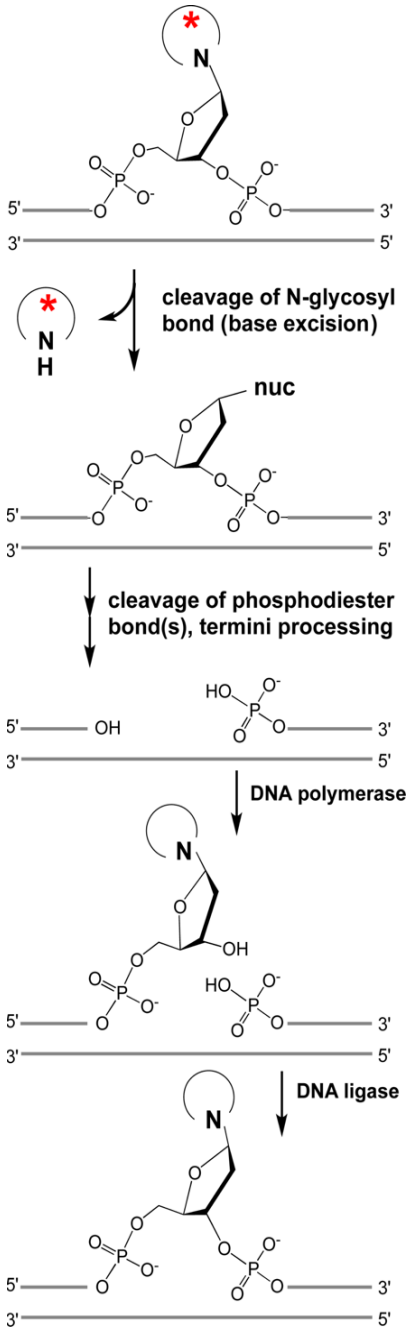


Figure 4 ***Chemical representation about principal BER steps***

After the cleavage of the N-glycosyl bond by specific DNA glycosylases, an AP endonuclease cuts the phosphodiester bond. The resulting gap is filled with new correct dNTPs by DNA polymerases and ligases. (Drohat and Coey, 2016)

Because of its complexity, different theories have been proposed to explain in detail how the BER pathway proceeds, including the “passing the baton” and “BERosome” models (Almeida and Sobol 2007; Allinson et al. 2004; Wilson and Kunkel 2000). A fine regulation exists among all the BER enzymes in order to coordinate every step of the DNA repair. This coordination is possible by means of several post-translational modifications (PTMs) able to regulate protein-protein interactions, pathway cascade signaling, cellular localization, conformational changes and protein stability (Almeida and Sobol 2007; Déry and Masson 2007). Moreover, several proteins, including p53 and Nucleophosmin, work as BER modulators (Fan and Wilson 2005; Vascotto et al. 2009).

Recently, other important non-canonical functions of the BER pathway, and particularly of BER proteins, have been described in literature.

In Drohat and Coey (and reference therein), a new surprising role of the BER, still completely unexplored, has been described. A well-studied epigenetic mark is the methylation of cytosine bases. Methylated bases are usually sited in repeatable CG dinucleotide sequences, called CpG islands, that are associated with gene repressing and chromatin remodeling. Specifically, the BER pathway can work as an epigenetic modulator through its “repair” ability in removing this epigenetic mark, by modulating the level of methylated cytosine and converting it back to unmodified cytosine (Drohat and Coey 2016).

Moreover, BER enzymes, such as APE1, PARP1 and SMUG1, possess non-canonical roles, not merely implicated in the BER. As it will be explained in next paragraphs, BER enzymes are also related to RNA metabolism, in transcriptional regulation of genes and also in miRNAs regulation (Antoniali, Lirussi, Poletto, et al. 2014; Ogawa and Baserga 2017; Poletto, Legrand, et al. 2016; Tell et al. 2009; Antoniali et al. 2017).

In next paragraphs, I will deeper focus on two main BER enzymes: DNA glycosylases and apurinic/ apyrimidinic endonucleases.

4.3.1 DNA glycosylases

Each one of the eleven *N*-glycosylase proteins present in mammalian cells is able to initiate the BER pathway after recognizing specific damaged bases. Once the recognition step is performed, its principal function is cleaving the damaged base leaving an apurinic/ apyrimidinic (AP) site. In a eukaryotic cell, several DNA glycosylases exist and they differ from each other for the mechanism of action, substrate specificity and excision kinetics (Sidorenko, Nevinsky, and Zharkov 2007; Sidorenko and Zharkov 2008; Takao et al. 1999). Although the existence of a high variety of glycosylases in mammalian cells, a significant redundancy in their damage selectivity is observed. For this reason, it has been observed that single knockout of one of them are not considered lethal *per se* (Wallace 2013).

Based on their mechanism of action, DNA glycosylases are classified in two groups: mono- and bi- functional glycosylases (Figure 5).

Monofunctional DNA glycosylases (e.g. uracil DNA glycosylases (UDG) including the mitochondrial uracil N glycosylases (UNG1), nuclear UNG2 and a single strand selective monofunctional uracil DNA glycosylases (SMUG1)) process the damaged DNA cleaving the C1-*N*-glycosidic bond and generating an AP site and the liberated nucleobase.

Bifunctional glycosylases, including the 8-oxoguanine DNA glycosylase (OGG1), NTH1 and NEIL family, display, in addition to their glycosylase activity, an additional AP-lyase activity (Svilar et al. 2011) (Figure 5). After the recognition of the oxidized base, bifunctional glycosylases use an amine nucleophile, such as a Lys side chain, to cleave the *N*-glycosidic bond, generating a Schiff base (imine) intermediate (Prakash, Doublié, and Wallace 2012). Subsequently, through their AP-lyase activity, bifunctional glycosylases cleave the DNA phosphodiester backbone on the 3' side of the lesion through a β-elimination resulting in a single strand break. A second cleavage on the DNA phosphodiester backbone is also possible on the 5' side of the lesion through δ-elimination (Bailly and Verly 1988) (Figure 5).

Usually, monofuctional glycosylases are implicated in the SP BER sub-pathway whereas the bifunctional in the LP BER sub-pathway (Dianov and Hübscher 2013).

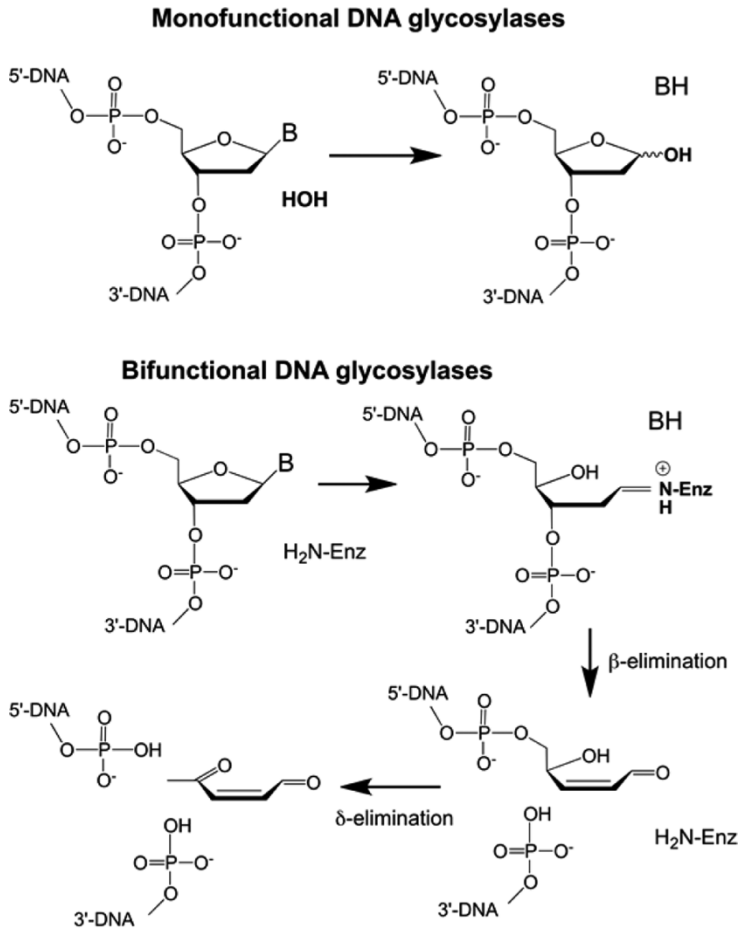


Figure 5 ***Mechanism of action of two types of DNA glycosylases in mammalian cells***

Mono- and bi- functional glycosylases differ from each other for the 5'-mojety generated. Monofuctional glycosylases cleave the N-glycosyl bond to the 5' side of the damaged base. Bifunctional glycosylases are also able to cleave the phosphodiester bond at the 3' side (β-elimination) in addition to a second cleavage at the 5' side (δ-elimination). (Drohat and Coey, 2016)

High expression levels of DNA glycosylases, as in the case of other BER enzymes, are distinctive of some types of cancer (An et al. 2007). The knowledge of different DNA glycosylases and their specific mechanism of action will allow the discovery of small molecule inhibitors of these proteins as potential therapeutic targets for treatment of several tumors (Donley et al. 2015).

4.3.1.1 8-oxoguanine glycosylase

Human 8-oxoguanine glycosylase (OGG1) is a bifunctional glycosylase working in the BER pathway. The 8oxoG- and FapyG- lesions, opposite to cytosine in the complementary strand, are recognized and removed by OGG1. OGG1 is able to cleave the *N*-glycosidic bond of 8-oxo-2'-deoxyguanosine. Moreover, belonging to the bifunctional subgroup, OGG1 is able to process oxidized bases through β-elimination mechanism and generates abasic sites that are subsequently processed by the final BER enzymes, polymerases and ligases (Kuznetsov et al. 2005). OGG1 belongs to the HhH-GPD superfamily containing conserved structural helix-hairpin-helix and GPD motifs (Yamagata 2001).

In the last decades, X-Ray crystallography studies have defined the structural binding between DNA and OGG1 (Kuznetsov et al. 2005, 2014; Rowland et al. 2014; Šebera et al. 2012). As Figure 6 shows, OGG1, during the recognition of the damaged base, forms non-specific electrostatic and hydrophobic contacts with the sugar-phosphate backbone kinking the DNA by 70°. After that, the oxidized G is fully flipped out from the DNA helix and deeply inserted into the active site of the enzyme. The cytosine located on the complementary strand and paired with 8oxoG starts hydrogen-bonding interactions with the amino acids Arg-154 and Arg-204 inserted into the DNA helix. After the flipping out of the oxidized G residue, amino acids Asn-149 and Tyr-203 are inserted into the abasic site (Kuznetsov et al. 2014). Although OGG1 establishes contacts also with undamaged G through its the active pocket site, it is able to discriminate and not process it (Fromme et al. 2003).

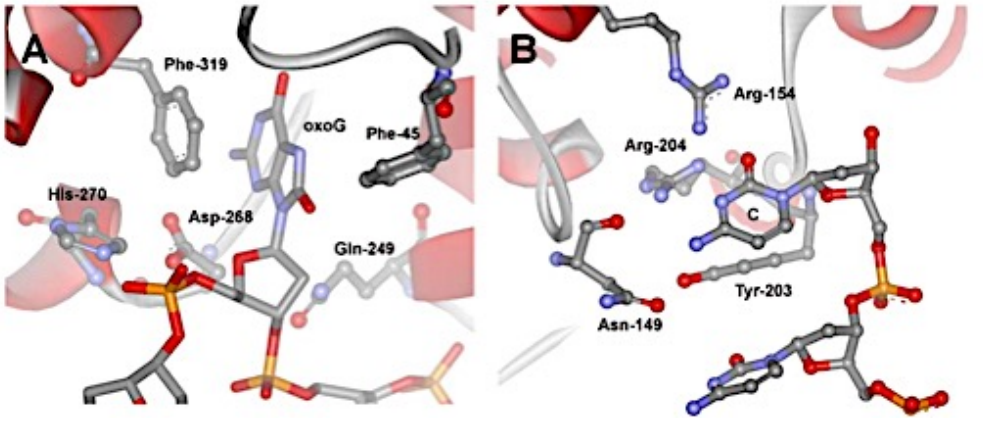


Figure 6 ***View of the catalytic pocket site of OGG1***

*First, specific amino acids contact the 8oxoG (**A**) and later with the cytosine opposite to the oxidized lesion (**B**). (Modified from Kuznetsov et al., 2014)*

4.3.2 AP endonuclease

Unrepaired abasic sites, generated by spontaneous hydrolysis of the DNA backbone or following processing by a glycosylase on a damaged base, result mutagenic and cytotoxic for the cell (Loeb and Preston 1986).

In order to maintain genome integrity, mammalian cells are endowed with specific enzymes called apurinic/apyrimidinic (AP) endonucleases. The importance of this enzymes is easily recognized in those cases in which deletions result in embryonic or early post-natal lethality (Iyama and Wilson 2013). In mammals, the major AP-endonuclease is the apurinic/apyrimidinic (AP) endonuclease 1 (APE1) (Demple, Herman, and Chen 1991; Hadi et al. 2002). Contrary to APE1, apurinic/apyrimidinic (AP) endonuclease 2 (APE2) is not involved in the repair of AP sites but is central for normal B cell development. APE2 ensures the activation of cytidine deaminase, enzyme needed for the expansion of germinal centers and regulating the antibody diversification (Guikema et al. 2011; 2014).

APE1 is a major enzyme of the BER pathway due to its importance in the correct progression of DNA repair (Tell et al. 2009, 1). Remarkably, as recently discovered, APE1 has several other activities besides its BER role (Antoniali, Malfatti, and Tell 2017) that will be further discussed.

The *ape1* gene is mapped on chromosome 14q11.2-q12 consisting of five exons and four small introns for a total of 2.6 kbp (Robson et al. 1992). Its transcription is regulated during the cell cycle with an increased transcription during the S-phase (Rivkees and Kelley 1994).

APE1 is a monomeric protein of about 36 kDa, composed of 318 amino acids, member of the homologous family comprising *E.coli* exonuclease III. APE1 is structured in α/β-sandwich globular fold coupled to an unstructured part composed of first 48 amino acids in the N-terminal (Gorman et al. 1997).

Two domains of APE1 can be functionally identified:

- the N-terminal domain (1-127) deputed to protein-protein interactions and RNA interaction, as well as the redox-dependent activity toward different transcription factors (Fantini et al. 2010, 1);

- the C-terminal globular region (residues 61-318), responsible for the endonuclease activity (Tell et al. 2009, 1).

APE1 undergoes different post-translational modifications *in vitro* as shown in Figure 7, although their effects *in vivo* are largely unknown.

Only a PTM on APE1 has been well described *in vivo* to be able to generate a truncated form of APE1. The first 33-amino acids located at the N-terminal domain can be cleaved by an unknown Granzyme-like activity (Yoshida et al. 2003). This mutant form lacks the nucleolar localization signal (NLS) sequences and its ability to interact with other proteins. The N-terminal domain can be subjected to phosphorylation affecting APE1 stability. Phosphorylation inactivates APE1 endonuclease activity (Huang et al. 2010; Busso, Lake, and Izumi 2010), while APE1 redox activity is increased. Another interesting modification of the N-terminal domain of APE1 is acetylation of different Lysins including Lys27-35 cluster. Positive charges of this acetylatable lysine residues of APE1 are essential for chromatin association. Their acetylation affects the ability of the protein to interact with NPM1 inhibiting APE1 accumulation into nucleoli (Lirussi et al. 2012; Busso, Lake, and Izumi 2010). Moreover, acetylation-mediated neutralization of the positive charges of the lysine residues induces a conformational change that enhances the AP endonuclease activity of APE1(Roychoudhury et al. 2017; Fantini et al. 2010). Acetylation of APE1 was detected in a subtype of breast cancer demonstrating how an aberrant modulation of APE1 has detrimental consequences on cell stability (Poletto et al. 2012).

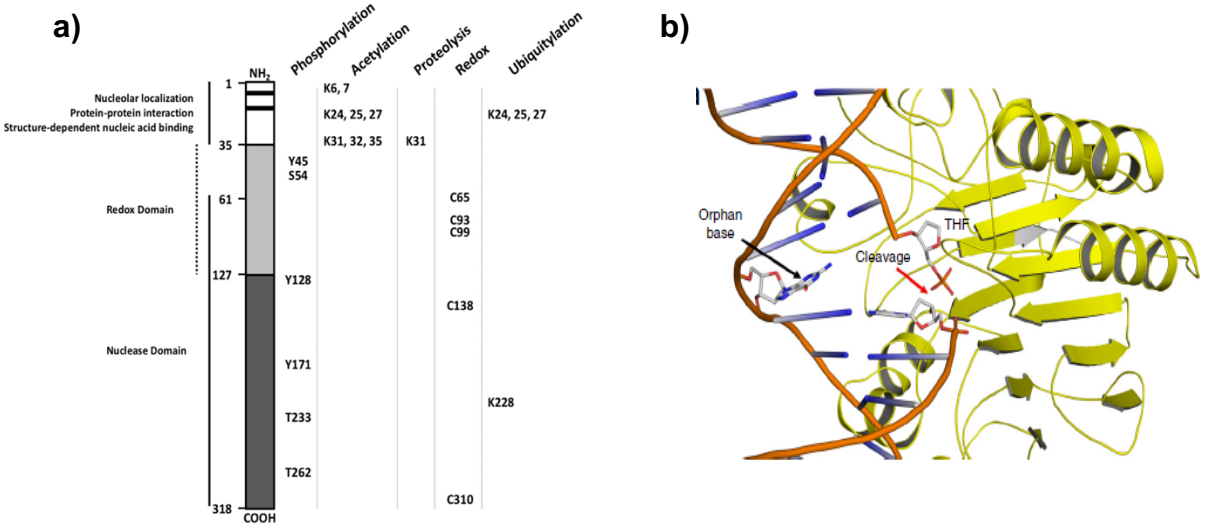


Figure 7 ***Structural and functional organization of APE1 protein***

a) *Each domain of APE1 has a specific function. Specifically, the N-terminal domain is deputed on nucleolar localization, protein-protein interaction, structure-dependent nucleic acid binding and redox activity. The C-terminal domain is deputed on the endonuclease activity. On the right side, amino acids essential for the relative activity are reported. (Poletto et al., 2016)* ***b)*** *Cartoon representation of the complex APE1:DNA product. APE1 is shown in yellow while the site of cleavage is indicated with a red arrow. THF indicates the remaining abasic site. (Freudenthal et al., 2015)*

APE1 possesses different nuclease activities on DNA as well as RNA. Regarding its role in the BER pathway, APE1 is able to cleave the DNA phosphodiester backbone to the 5' side of abasic sites. The cleavage produces SSB with a 3'-hydroxyl (OH) and a 5'-deoxyribose phosphate ends (dRP). Subsequent BER enzymes are deputed to filling and sealing the gap.

The active site of the endonuclease activity is defined by several residues including His-309, Glu-96, Asp-283, Thr-265, Tyr-171, Asn-68, Asp-210, Asp-70 and Asn-212, most of them are involved in a hydrogen bonding network formation (Beernink et al. 2001; Mol et al. 2000; Freudenthal et al. 2015a). After the abasic site recognition, APE1 discriminates the lesion, excluding any normal dNTP, and stabilizes an extra-helical DNA backbone distorted about 35°.

An important characteristic of APE1 activity is its dependence on magnesium ions. Upon formation of the initial recognition APE1-DNA complex, an additional rearrangement is needed to allow the efficient execution of the hydrolytic reaction. The presence of Mg^{2+} (or Mn^{2+}), positioned in the active site E96 is important to promote this rearrangement (Erzberger and Wilson 1999). As Figure 8 shows, the cleavage reaction, metal ion-dependent, needs also a molecule of water acting as a nucleophile (Freudenthal et al. 2015a).

Moreover, although replacement of Asp-90, Asp-308 or Glu-96 by alanine does not significantly compromise nucleic acid binding, a reduction in enzymatic activity is observed (Barzilay et al. 1995).

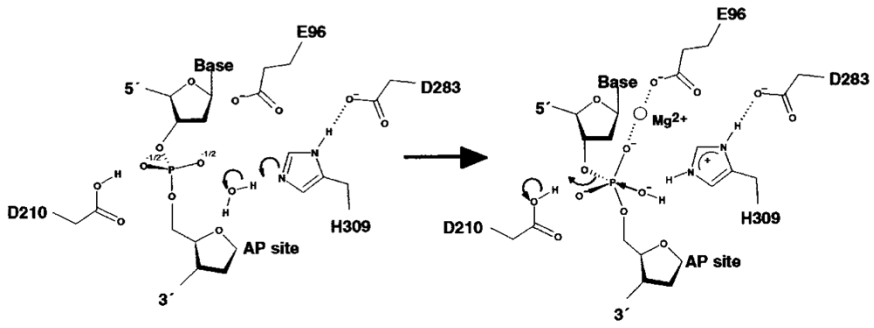


Figure 8 ***Catalytic mechanism of APE1 processing when an AP site is present in DNA***

After a first DNA recognition, APE1 is able to cleave in a Mg^{2+} dependent reaction the abasic site. E96, catalytic site of the protein, is fundamental to promote the cleavage reaction. (Erzberger and Wilson, 1999)

An alternative pathway of BER, in which APE1 has a fundamental role as endonuclease, is the Nucleotide Incision Repair (NIR) pathway.

NIR pathway works as a backup of BER when glycosylases are absent or inactive on the damage, thus insuring a correct removal of damaged bases as a result of oxidative stress (Gros 2004a; Timofeyeva et al. 2011; Ishchenko et al. 2006; Gelin et al. 2010; Daviet et al. 2007a; Redrejo-Rodríguez et al. 2016). In that pathway, APE1 processes directly at the 5' of the oxidized base without the intervention of a glycosylase leaving fragments with 3' OH extremities (Gros 2004a). After the removal of the dangling damaged nucleotide by FEN1 (Kim, Biade, and Matsumoto 1998; Klungland and Lindahl 1997), the DNA backbone can be efficiently repaired by DNA polymerases. In this way, the NIR action avoids the generation of potentially toxic AP-intermediates (Ischenko and Saparbaev 2002).

Several works have focused their attention on the NIR activity of APE1, by defining the substrates that are efficiently processed by APE1 including 5,6-dihydro-2'-deoxyuridine (DHU), 5,6-dihydrothymidine (DHT), 5-hydroxy-2'-deoxyuridine (5OHU) (Gros 2004a; Ischenko and Saparbaev 2002), 5-hydroxy-2'-deoxycitidine (5OHC) (Daviet et al. 2007a; Gros 2004a; Ischenko and Saparbaev 2002), alpha-2'-deoxynucleosides (αdA, αdT and αdC) (Ide et al. 1994; Gros 2004a), the majority of which are generated under ionizing radiation and certain drugs. This non-canonical activity of APE1 could explain how the lack of DNA glycosylases does not render cells or mice more sensitive

to oxidative agents and IR (Alseth et al. 1999; Blaisdell and Wallace 2001; Friedberg and Meira 2003). This is in contrast to what is observed in APE1 deficient cells (Ludwig et al. 1998; Ramotar et al. 1991; Cunningham et al. 1986). In fact, the haploinsufficiency in AP endonuclease activity in mice leads to reduced survival associated with an increased cancer susceptibility when exposed to oxidative stress (Meira et al. 2001).

Moreover, what emerged is that the NIR activity of APE1 can be observed under environmental conditions dramatically different in terms of salts, pH and structural involvement compared to the classical BER activity. The optimal conditions for NIR activity are very similar to 3' → 5' exonuclease APE1 activity, characterized by a pH around 6.4 - 6.8, and a KCl concentration of 50 mM (Gros 2004a). Moreover, NIR is more active at 100-fold lower $MgCl_2$ concentration compared to AP-endonuclease activity (Gros 2004a). Interestingly, the N-terminal domain of APE1 deputed to protein-protein interaction and indispensable to redox activity but not AP-endonuclease activity (Izumi et al. 2005a), is, on the contrary, essential for the NIR activity (Gros 2004a). Moreover, Timofeyeva *et al.* demonstrated that Lysine in position 98 contributes significantly in the 5' - phospodiester bond hydrolysis of DNA substrate, but not to the dissociation of the enzyme from the product complex (Timofeyeva et al. 2011). The substitution of this amino acid influences the NIR activity more than BER, demonstrating that the active site of APE1 involved in NIR and BER pathways is the same, but different conformations of it are responsible for the incision of unrelated lesions as AP sites and DHU, substrate of BER and NIR, respectively (Timofeyeva et al. 2011).

APE1 is also active on damaged single stranded DNA suggesting a new role in transcription, replication and/or recombination (Marenstein, Wilson III, and Teebor 2004). APE1 has DNA 3'-phosphatase and has a 3'-5' DNA exonuclease activity *in vitro* on mismatched deoxyribonucleotides at the 3' termini of nicked or gapped DNA (Chou and Cheng 2003; Wilson 2003; Izumi et al. 2005b). Again, for the exonuclease activity of APE1, specific saline conditions are essential (Chou and Cheng 2003).

Moreover, a lot of data supports the role of APE1 in the RNA metabolism (Tell, Wilson, and Lee 2010) including:

- APE1 cleaves AP-site containing single stranded RNA in *in vitro* assay (Berquist, McNeill, and Wilson 2008);
- APE1 physically associates with ribosomal RNA (rRNA) allowing its correct processing (Vascotto et al. 2009);
- APE1 interacts with a wide variety of proteins involved in rRNA processing, including Nucleophosmin (NPM1) (Vascotto et al. 2009).

  It has been proposed that APE1/NPM1 interaction inhibits APE1 binding to RNA through direct competition. At the same time, NPM1, binding rRNA, is able to mask rRNA from APE1. In tumoral cells, in which APE1 and NPM1 levels and the interaction among them are higher than in healthy cells, APE1/NPM1 inhibitors, characterized in our laboratory, could be a chance in order to impair this interaction (Poletto, Malfatti, et al. 2016). Other APE1 interactors involved in RNA metabolism are YB-1 (Chattopadhyay et al. 2008, 1) and hnRNP-L (Kuninger et al. 2002).
- As other members of exonuclease III family, APE1 conserves the RNase H activity which allows the degradation of the RNA strand of a DNA-RNA duplex *in vitro*. Several residues are essential for this activity, including Asp-219, important for RNA and DNA enzymatic activity and binding, and Asp-90, Asp-308 and Glu-96 involved in RNA and DNA enzymatic activity but not in the binding activity (Barzilay et al. 1995).To date, the biological effect of this function is still unknown.
- The catalytic site of the endonuclease activity of APE1 has an interesting endoribonuclease function of APE1 (Barnes et al. 2009; W.-C. Kim et al. 2011) that preferentially cleaves at UA, UG and CA sites in single-stranded regions of RNAs. Amino acids involved in the endoribonuclease activity are several including: N68, D70, Y171, D210, F266, D308 and H309. *In vitro* experiments, obtained by Barnes *et al*., have demonstrated that this function is explicated on the proto-oncogene c-*myc* RNA, feature of the development of several tumors. APE1 is able to cleave, via its endoribonuclease activity, a specific coding region of c-*myc* mRNA *in vitro.* It was demonstrated that APE1 regulates the stability of c-*myc* mRNA in cells. Moreover, upon knockdown of endogenous APE1, c-*myc* mRNA is specifically destabilized demonstrating that APE1 may control c-*myc* mRNA level and half-life *in vivo* (Barnes et al. 2009).

- APE1 has a weak 3'-5' exoribonuclease activity (Chohan et al. 2015) removing a phosphoryl group from the 3' end of RNA decay products. An interesting data on RNA nuclease activities of APE1 is that the protein does not require Mg^{2+}.
- Finally, recently discovered in our laboratory, APE1 has a role in pri-miRNA processing and stability via association with the DROSHA-processing complex during genotoxic stress (Antoniali et al. 2017).

Finally, other important functions of APE1, different from its nuclease activities, regard its ability to work as a redox co-activator of several transcription factors that regulate gene expression, involved in cancer promotion and progression. Indispensable for redox function is the residue of cysteine in position 65. APE1 is able to control the transcriptional regulation of gene expression by keeping, in a reduced state, critical Cys residues of some transcription factors. The redox modulation enhances the DNA binding activity of several transcription factors including AP-1, p53, HIF-1alpha, NF-KB, CREB and Egr-1 (Tell et al. 2009, 1; Cesaratto et al. 2013; Fantini et al. 2008, 2010).

Moreover, another still not-well characterized function of APE1 is its transcriptional repressor activity through binding to the negative calcium responsive elements (nCaRE). This function is promoted by PTH and the same APE1 promoters (Antoniali, Lirussi et al. 2014).

Several clinical studies have observed that APE1, as well as other BER proteins, is involved in several types of cancer. For this reason, APE1 represents a good candidate as chemotherapeutic target (Wilson and Simeonov 2010; Poletto, Malfatti, et al. 2016; Rai et al. 2013, 2012).

As above anticipated, enzymes merely deputed to DNA repair, such as APE1, have also a role in RNA metabolism (Antoniali, Malfatti, and Tell 2017). For this reason, next paragraphs focus on the description of a relative new type of lesion involving RNA underlining the hypothesis of a role of APE1 in its processing.

4.4 Incorporation of ribonucleotides into DNA

RNA:DNA hybrids are particular structures in which an RNA molecule is paired with a DNA molecule to form a precise configuration. Generation of these structures occurs physiologically in cells during processes such as DNA replication, DNA transcription, retroviral infection, retro-element mobilization and telomere elongation (Förstemann and Lingner 2005). If not properly processed, the presence of RNA:DNA hybrids induces harmful consequences to the genome integrity causing reducing fork speed with a replication-transcription collisions and chromosomal break repair (Hamperl and Cimprich 2014; Brambati et al. 2015).

At least three types of RNA:DNA hybrids can be found in eukaryotic cells, which are represented in Figure 9:

- *ssRNA paired with ssDNA*, in which a single strand molecule of RNA is complementary to a single molecule of DNA*;*
- *R-loop*, in which the two DNA strands are separated with only one hybridized to RNA while the other is single stranded DNA*;*
- *rNMPs embedded in DNA*, when a single, or more, ribonucleotide(s) is/are embedded in a double stranded molecule of DNA.

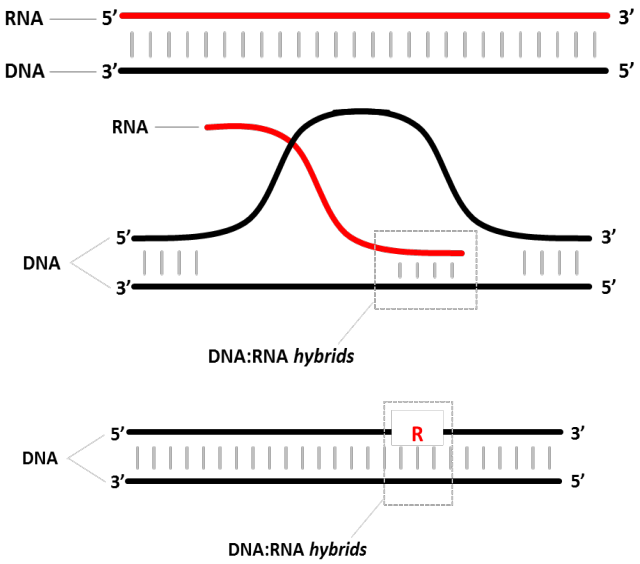


Figure 9 ***Schematic representation of three RNA:DNA hybrids observed in eukaryotic cells***

ssRNA paired with ssDNA (upper), R-loop (middle) and single or more rNMPs embedded in DNA (bottom) are the best-represented form of RNA:DNA hybrids.

Although undesirable, rNMPs incorporation in DNA is a very frequent event, so that it is considered the most common type of 'DNA damage' occurring in normal cells (Williams and Kunkel 2014; Koh, Balachander, et al. 2015).

For this reason, in next paragraphs of this Thesis, the attention will be mostly focused on the rNMPs incorporation into DNA elucidating causes, repair, and inefficient repair-consequences of this phenomenon.

4.4.1 Causes of rNMPs incorporation within DNA

Starting from quantification data of the nucleotides rate into the cell, it was demonstrated the the rNTPs pool far exceeds the dNTPs pool under basal conditions (Koh, Balachander, et al. 2015; McElhinny et al. 2010) (Figure 10). Although the dNTP: rNTP ratio is variable, the amount of rNTPs is greater than the amount of dNTPs between organisms and tissues (Nick McElhinny et al. 2010; Kennedy et al. 2010). The amount of rNTPs in *S. cerevisiae* is generally 40–300 - fold higher than that of dNTPs in cycling cells (Nick McElhinny et al. 2010; Crespan et al. 2016; Traut 1994) increasing the probability of incorrect rNMP incorporation during DNA replication and repair (Koh, Balachander, et al. 2015; Clausen et al. 2013; Nick McElhinny et al. 2010; Crespan et al. 2016).

The greater amount of rNTPs compared to dNTPs, coupled with an imprecise activity of replicative polymerases (Koh, Balachander, et al. 2015; Clausen et al. 2013; Nick McElhinny et al. 2010; Crespan et al. 2016), would explain its high frequency, about more than 100 million rNMPs in mammalian DNA, transiently incorporated during each replication cycle. Replicative DNA polymerases carry an active site in which a bulky amino acid residue at the entry site discriminates dNTPs from rNTPs (Joyce 1997). DNA polymerases possess a 3'-exonucleolytic proofreading activity that, in addition to the DNA Mismatch repair (MMR) pathway, ensures a correct discrimination of the four different bases lowering the error rate to around 10^{-10} (Kunkel 2009). In this way, DNA replication proceeds with high fidelity.

Thus, DNA polymerases are able to well discriminate rNTPs. Under conditions in which the rNTPs: dNTPs ratio is in favour of rNTPs, DNA polymerases can erroneously incorporate rNTPs than dNTPs (Joyce 1997;

Brown and Suo 2011). It has been estimated the potential rate of incorporation by DNA Polymerase α (around 1'900 rNMPs), DNA Polymerase ε (around 9'600 rNMPs) and DNA Polymerase δ (around 2'200 rNMPs) (Nick McElhinny et al. 2010; Marasco et al. 2017). Remarkably, the exonuclease activity results inefficient *versus* this type of error (Figure 10).

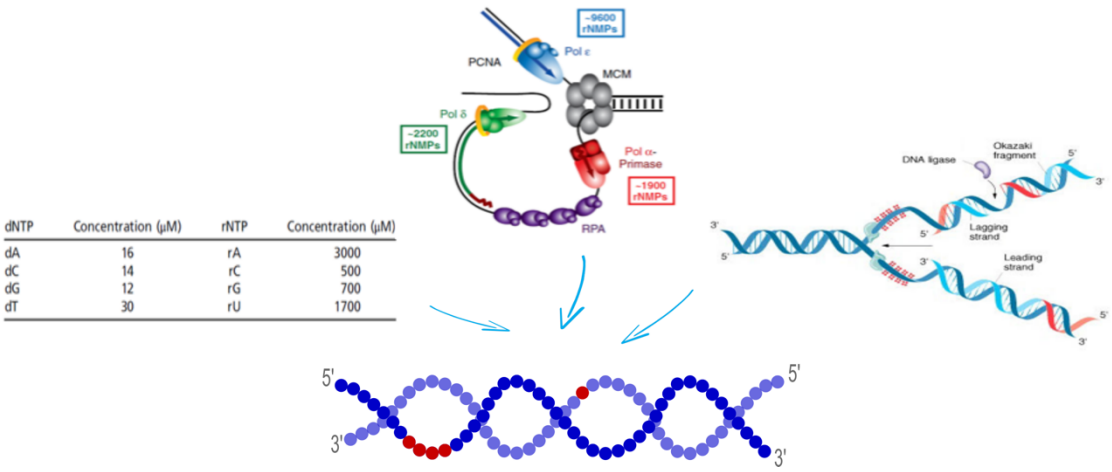
Further, if a damaged or a wrong base is incorporated into DNA, the replicative fork is stopped. To counteract the deleterious effects of the replicative DNA polymerase stalling, cells possess a specialized system called **t**rans**l**esion DNA **s**ynthesis (TLS) bypass. In this pathway, translesion polymerases that have a large active site allow lower base and sugar selectivity allowing to bypass the damage. Again, under conditions in which the amount of rNTPs is much higher than dNTPs into the cell, the risk of mis-incorporation increases. This mechanism, if on one hand, contributes to cell survival promoting DNA replication, on the other may fix a damage/mutation (Sassa et al. 2016).

One additional source of rNMPs in DNA may derive from the mechanism of replication of the DNA lagging strand. In order to initiate, DNA polymerases need a 10-ribonucleotides primer, synthetized by a RNA primase. Starting from this primer, a DNA fragment of about 200 nucleotides is synthetized, called Okazaki fragments (Clausen et al. 2013). Subsequently, RNA primers are duly removed to allow the filling of the DNA gap and the generation of a continuous DNA strand (Rossi and Bambara 2006). If these primers are not completely eliminated, isolated rNMP/s can permanently persist within genomic DNA (Figure 10).

Recently, it has been found that rNMPs are also incorporated by DNA polymerase γ in mitochondrial DNA, in heavy- as well as light-strand DNA. Increased levels of embedded ribonucleotides, affecting mtDNA stability and impairing new rounds of mtDNA replication, may contribute to a new pathogenic mechanism (Berglund et al. 2017). Further studies are needed regarding the consequences of the rNMPs incorporation in mtDNA and their repair processing.

Finally, although not fully explored yet, generation of hydroxyl radicals from oxidative stress could be the cause of conversion of the deoxyribose sugar into ribose *in vitro* and *in vivo* (Randerath et al. 1992). This can happen both in the cellular nucleotide pool or directly into the DNA.

Additional data are needed in support to this hypothesis.



dNTP	Concentration (μM)	rNTP	Concentration (μM)
dA	16	rA	3000
dC	14	rC	500
dG	12	rG	700
dT	30	rU	1700

Figure 10 ***Major causes of rNMP incorporation within DNA***

Schematic representation of a double helix of DNA (blue dots) in which one or more ribonucleotides (red dots) are embedded in DNA. Different are the causes of rNMPs incorporation in DNA including, starting from left, high levels of rNMPs compared to dNMPs in the cellular nucleotide pool, inefficiency into sugar discrimination by several DNA Polymerases (middle), and finally residue rNPMs from RNA primers used to generate Okazaki fragments (right). (Modified from Nick McElhinny et al., 2010 and Griffiths et al., 2010)

4.4.2 Effects of rNMPs incorporation into the DNA

Being a relative new discover in the DNA repair field, studies regarding the consequences of rNMP incorporation in DNA are not abundant and the majority of them are conducted only in yeast models.

Considering that the difference between rNTPs and dNTPs is the presence of hydroxyl (OH) group in 2' position of the sugar, when a rNTP is erroneously embedded into DNA, its presence highly destabilizes the DNA backbone.

Several studies were carried on in order to try to better explain the effects of this lesion on DNA. It has been demonstrated that the presence of one or more rNMPs increases the susceptibility to DNA hydrolysis, and strand

cleavage, under basal conditions (Chiu et al. 2014). From data obtained by Atomic Force Microscopy (AFM), X-ray crystallography and high-resolution NMR solution structures, it was demonstrated that this lesion alters DNA elasticity and structure in a sequence dependent manner. Indeed, the lesion locally perturbs the structure asymmetrically on the 3' side of the lesion in both the riboguanosine-containing and the complementary strand of the duplex (Chiu et al. 2014; Koh, Chiu, et al. 2015; Evich et al. 2016). Moreover, it has been reported that the presence of a single rNMP in DNA changes the global conformation of DNA from B-form to A-form DNA, although it is dependent on the size, sequence and crystallization conditions (Ramakrishnan and Sundaralingam 1993a, [b] 1993). Finally, it affects the activity and function of several DNA-interacting proteins increasing the DNA fragility and mutability (Williams and Kunkel 2014; Caldecott 2014).

Recently it has also seen that rNMPs in DNA act as template for DNA synthesis (Storici et al. 2007; Shen, Nandi, et al. 2011), although the DNA polymerases processivity on rNMP tracts is reduced (Storici et al. 2007).

4.5 Ribonucleotide Excision Repair pathway

Since the presence of ribonucleotides into DNA affects genomic stability, eukaryotic cell needs the action of enzymatic pathway deputed to rNMPs removal called Ribonucleotide Excision Repair (RER) pathway (Reijns et al. 2012) (Figure 11).

The first and main enzyme acting in the RER pathway is RNase H2, a nuclear enzyme that cleaves at a single, or more ribonucleotides embedded in DNA. RNase H2 incises the DNA backbone on the 5'-side of the ribonucleotide. Although RNase H1 is a protein similar to RNase H2, it is not active on a single rNMP within DNA and it is not able to promote the starting of RER pathway (Sparks et al. 2012). Then, flap endonuclease (Fen1) incises 3' side of the rNMP to release it, leaving a gap that will be filled by DNA Polymerase δ or ε and ligated by Ligase I (Sparks et al. 2012; Rydberg and Game 2002) (Figure 11).

As happens for the BER, all RER enzymes also work in a coordinate way in order to guarantee the correct repair of the damaged DNA. Moreover, all of the four enzymes working in RER pathway show interactions with PCNA which has a stimulating functional role on these enzymes (Sparks et al. 2012; Bubeck et al. 2011).

Surprisingly, the RER pathway is highly conserved from Eukaria to Archea to Bacteria (Heider et al. 2017). An interesting difference is about RNase HII, the prokaryotic corresponding protein of the eukaryotic RNase H2. RNase HII is in monomeric form in Archea and Bacteria differently from the trimeric RNase H2. Although not still well demonstrated, the role of the three subunits composing the eukaryotic RNase H2 is associated with other more important biological functions of the protein.

Published data have observed that some effects of the RER inactivity, principally due to RNase H2 defects, result in S-phase checkpoint activation (Williams et al. 2013) and slow cell growth (McElhinny et al. 2010). Moreover, as expected, a lot of rNMPs are left unrepaired into the genome.

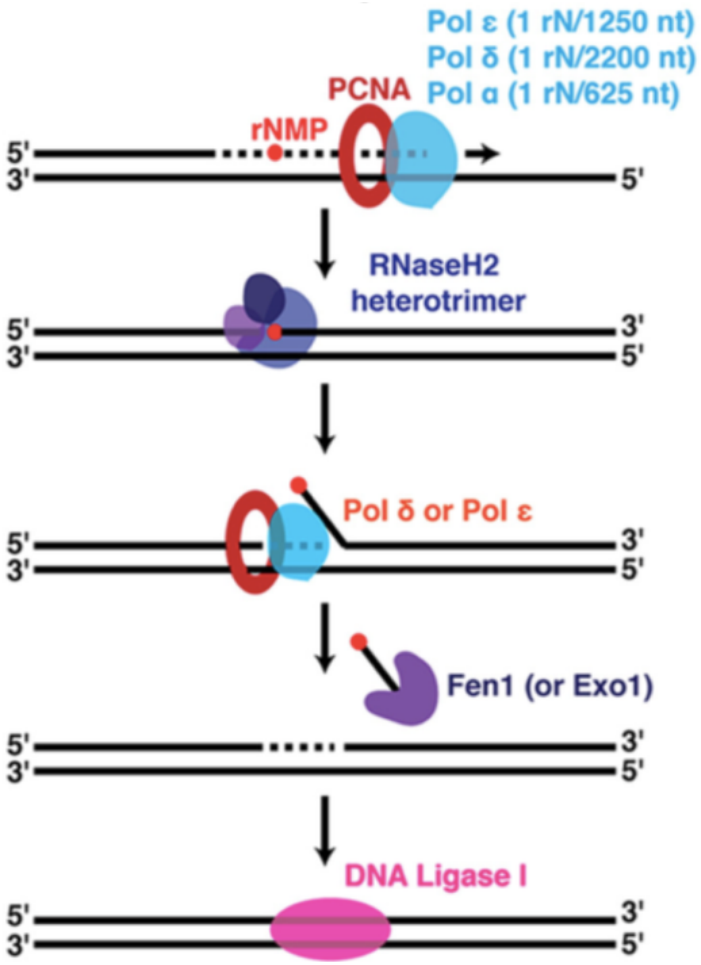


Figure 11 ***Principal steps of Ribonucleotide Excision Repair Pathway***

rNMP is specifically recognized and incised by RNase H2 protein. The synchronized work of Polδ or ε, Fen1 and Ligase I allow the complete repair of the damage. (Heider et al., 2017)

In next paragraphs, a deeper explanation about the structure and function of RNase enzymes will be done with a brief remark about pathologies associated to RNase H2.

4.5.1 Ribonuclease H enzymes

Generally, DNA:RNA hybrids are efficiently processed by a class of enzymes called RNase H, a family of endoribonucleases able to cleave the RNA strand in a sequence-non-specific manner producing short oligonucleotides and generating 5'-phosphate and 3'-hydroxy free groups. At the same time, a single strand DNA is left and used as a template to reconstitute the double strand DNA (Ohtani et al. 1999).

Eukaryotic RNase H family includes two classes of enzymes: RNase H1 (type 1) and RNase H2 (type 2). As will be addressed in the next paragraphs, these two proteins differ from each other by amino acids sequence and biochemical properties. On the contrary, they partially share the substrate specificity. Indeed, they are able to cleave at the 5' side of RNA phosphodiester bonds (Nowotny et al. 2007; Chapados et al. 2001), using bivalent cations such as Mg^{2+} and Mn^{2+} (Hausen and Stein 1970) (Figure 12).

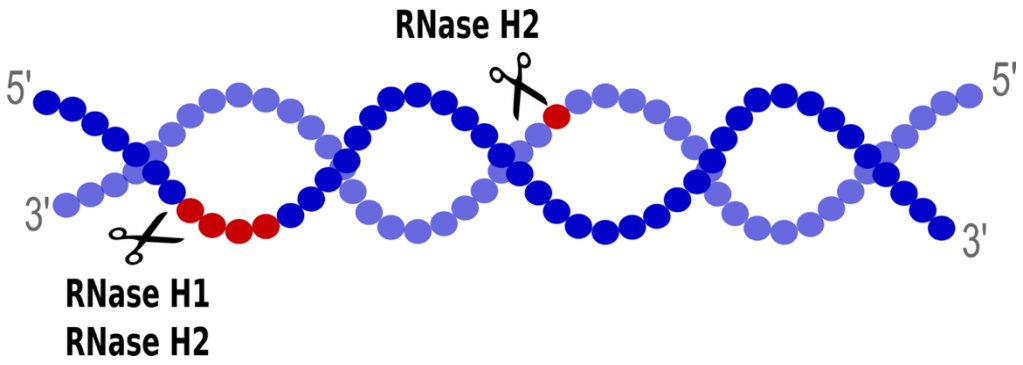


Figure 12 ***Activity of RNase H enzymes on DNA***

Schematic representation of a double helix of DNA (blue dots) in which one or more ribonucleotides (red dots) are embedded in DNA. Scissors indicate RNase H proteins. RNase H1 is able to cleave at the 5'side of at least four rNMPs embedded in DNA whereas RNase H2 also cleaves at the 5'side of a single rNMP embedded in DNA.

4.5.1.1 RNase H1

Human RNase H1 is a monomeric protein composed of 286 aminoacids with a molecular weight (MW) of about 32,200 Da.

RNase H1 is homologous to the prokaryotic RNase HI and the RNase H domain of retroviral reverse transcriptase (Cerritelli and Crouch 2009). Its localization is mostly nuclear, although its function, in this compartment, is still not understood. Moreover, when localized into the mitochondria, RNase H1 exerts an essential role in the mtDNA replication.

The typical organization of eukaryotic RNase H1 consists of different domains each having a specific function. First, the most important domain is exerted by the H-Domain (RNase **H Domain**) localized at the C- terminal. Through this domain, RNase H1 is able to process different RNA: DNA hybrids structures including R-loops and tract composed of at least four ribonucleotides embedded in DNA. The prerogative of this function, is the recognition of both DNA:RNA hybrid strands (Lima et al. 2007). Its ability to bind various nucleic acids is mediated by the HBD (**H**ybrid **B**inding **D**omain), localized at the N-terminal. Upstream of the HBD, many, but not all, eukaryotic RNase H1 possess an MTS (**M**itochondrial **T**argeting **S**equence), required for protein localization within mitochondria. RNase H1 works by removing RNA primers used for mtDNA replication (Cerritelli et al. 2003). As demonstrated, *rnaseh1* null mouse embryos arrest development due to a failure into the amplification of mitochondrial DNA (mtDNA). Finally, the CD (**C**onnection **D**omain), a linker sequence between the N-terminal HBD and the C-terminal RNase H domain allows the N- and C-terminal regions to move rather freely in and around the substrates. This region can be variable in length and composition, suggesting a putative role in the protein-protein interaction.

4.5.1.2 RNase H2

RNase H2, differently from the monomeric prokaryotic RNase HII, is a heterotrimeric protein including three subunits, subunit A, around 299 aminoacids (MW ≈ 33,400), subunit B, around 308 aminoacids (MW ≈ 34,800) and subunit C, of 164 aminoacids (MW ≈ 17,800) (Cerritelli and Crouch 2009; Jeong 2004) (Figure 13).

Although sharing with RNase H1 the same endoribonuclease function on degrading single stranded RNA in R-loop structures, the function of

cleaving the 5' side of a single mis-incorporated rNMP present in a DNA molecule belongs only to RNase H2 (Hiller et al. 2012; Cerritelli and Crouch 2009).

The ability of the eukaryotic RNase H2 to cleave mis-incorporated rNMPs is attributed to its catalytic domain, i.e. subunit A. It has been demonstrated that subunits B and C form a complex that may serve as a nucleation site for the subunit A to form the catalytic active protein, or for possible interaction with other protein partners to support other functions. Thus, although the exact function of the accessory components, subunits B and C, are not still fully understood, their presence may confer important roles to the whole protein complex. In support of this, published data have demonstrated that RNase H2 interacts with PCNA (Meslet-Cladiére et al. 2007; Chon et al. 2009). Specifically, subunit B possesses a specific motif defined by the sequence QxxmxxΦΦ where x is any residue, m is aliphatic hydrophobic and Φ is an aromatic called PIP box (PCNA-interacting peptide). The PIP box, localized at C-terminus of the protein, is required for the interaction of RNase H2 with PCNA, a fundamental protein that clamps around double strand DNA during DNA replication allowing the transport of elongating polymerases and other factors involved in Okazaki fragment processing to the replication fork. The interaction between RNase H2 and PCNA mediated by PIP sequence on the subunit B leads to an exclusive RNase H2 localization at replication foci (Bubeck et al. 2011). It is not fully known if the interaction with PCNA is limited to the RNase H2 localization to the replication foci or involved other functions. Moreover, the consequences of this interaction are not still completely understood.

Although it has been demonstrated that this interaction does not affect the catalytic activity of RNase H2 *in vitro* (Bubeck et al. 2011; Chon et al. 2009), it opens new issues about the involvement of RNase H2 in replication/repair processes.

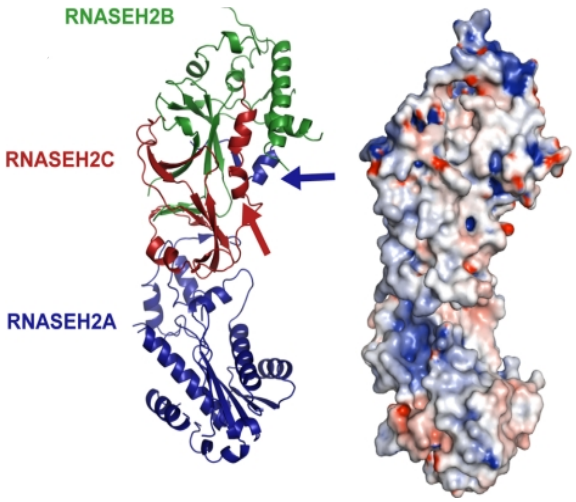


Figure 13 ***Representation of the subunits A, B and C, components of the eukaryotic RNase H2***

The core of the catalytic domain (RNASEH2A; blue) is stacked on the interwoven auxiliary RNASEH2B (green) and RNASEH2C (red) subunits (left). RNase H2 structure colored following the electrostatic surface potential (+9kT/e to −9 kT/e; electropositive in blue and electronegative in red) on the solvent accessible surface (right). (Reijins et al., 2011)

4.5.2 Pathologies associated with RNase H2 protein

The essential role of RNase H2 in cell viability is demonstrated by data in which defects in RNase H2, that abolish the enzymatic activity, result embryonically lethal in mice (Reijns et al. 2012; Hiller et al. 2012). For this reason, very little is known about a putative association between RNase H2 defects and disorders/disease.

From the literature, it is well known that RNase H2-null murine embryonic fibroblasts (MEFs) activate a p53-dependent damage response (Cerritelli and Crouch 2009). Same results were observed also in humans: specifically, when RNase H2 is partially absent or catalytically-inactive, the cells arrest in S and G2/M phases activating the DNA damage response (DDR). Moreover, a high rate of rNMPs, incorporated into DNA, has been detected causing a consequent block of the DNA replication (Lazzaro et al. 2012; Pizzi et al. 2014).

Interesting results have underlined the association between *biallelic* mutations in each of the three subunits of RNASE H2 and the onset of an inflammatory disorder called Aicardi Goutières syndrome (AGS) (Crow et al. 2006) (Figure 14).

AGS is a rare congenital encephalopathy with an autosomal recessive hereditary phenotype and symptoms similar to those of congenital viral infection (Crow et al. 2006; Rice et al. 2007). The signs observed in patients are several, including: cutaneous symptoms, acquired microcephaly, brain white matter abnormalities, intracranial calcifications and an elevated number of white cells (Crow and Manel 2015).

It has been well-defined how AGS shares features with the systemic lupus erythematosus (SLE), a complex autoimmune pathology characterized by the presence of antibody direct against antigens, including nucleic acids. In SLE pathologies, including AGS, an increase of antiviral type I IFN levels in the cerebrospinal fluid stimulates loss of B cells self-tolerance with a following production of autoantibody. After antibody-antigen recognition, immune complexes are generated with a consequent deposit in the capillary (Pendergraft and Means 2015).

Several genes, encoding for nucleic acid repair enzymes, result mutated in AGS including TREX1, SAMHD1, ADAR1 and RNASEH2 (Figure 14). Altered RNase H2 function in AGS patients may result in increased level of rNMPs in DNA which induces a innate immune response through the IFNγ signaling and genomic instability with activation of the DNA damage response signaling (Brzostek-Racine et al. 2011; Reijns et al. 2012; Pizzi et al. 2014). Moreover, the interesting observation that mutations found in the accessory subunits B and C are linked with AGS pathology would explain their importance in the protein complex (Chon et al. 2009) (Figure 14).

Finally, a screening of gastric cancers has found different mutations in different genes coding for several proteins involved in protein synthesis, genomic stability maintenance, metastasis, metabolic improvement, cell signaling pathways and chemoresistance. Among them, mutations in the gene coding for the subunit B of RNase H2 has emerged (Mottaghi-Dastjerdi et al. 2015). The association between mutated RNase H2 and cancer needs further study.

Further studies are ongoing to better understand the multiple roles of RNase H2 in DNA replication/repair, and genome integrity. Further studies are also needed regarding its pathological implications with the aim of finding therapeutic strategies.

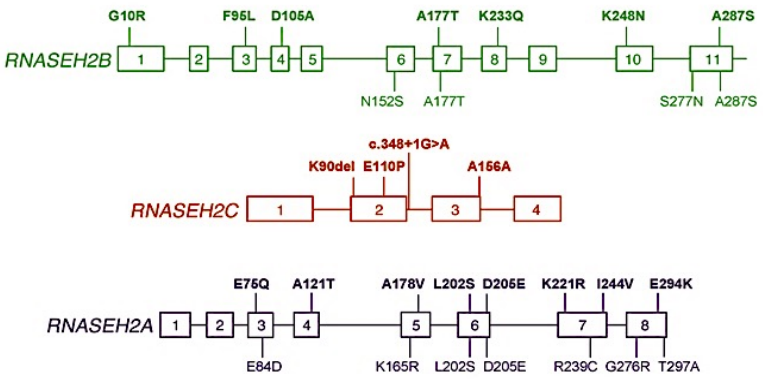


Figure 14 ***RNASEH2 mutations promoting systemic autoimmunity***

Polymorphisms in all the three subunits composing RNase H2 identified in individuals with SLE are shown in bold above. Below are indicated the variants occurring in control population. (Günther et al., 2015)

4.6 Different repair systems of rNMPs embedded in DNA

A still open question is whether other DNA repair systems, other than the RER pathway, may remove rNMPs embedded in DNA (Williams et al. 2013; Williams, Lujan, and Kunkel 2016).

Trying to answer to this issue, data published have discovered that enzymes belonging to DNA repair pathways can work as a back-up mechanism when the RER mechanism is inefficient. Among them, Topoisomerase I (Top1) can compensate RNase H2 deficiency (Williams et al. 2013; Sekiguchi and Shuman 1997). Top1 is an essential enzyme deputed to resolve DNA supercoils generated during replication and transcription (Capranico, Marinello, and Chillemi 2017). It was demonstrated that Top1 is able to cleave at the 5'-side of rNMPs (Williams et al. 2013) generating as DNA ends, 5'-OH and cyclic 2'-3' phosphate termini. Then, the cleavage is followed by nick processing by Srs2–Exo1 (Potenski et al. 2014; Kim et al. 2011).

A role for the Nucleotide Excision Repair (NER) pathway has been also hypothesized. This pathway generally excises bulky and non-bulky DNA base adducts (Reardon and Sancar 2005). Because of, the rNMPs incorporation distorts the DNA backbone, NER enzymes could be a good candidate in repairing this type of damage.

Data obtained from Bacteria show an involvement of NER factors in the removal of rNMPs in DNA (Vaisman et al. 2013; Lindsey-Boltz et al. 2015), contrarily to what is observed for human NER factors which are not involved in rNMPs repair (Lindsey-Boltz et al. 2015). Evidences about this difference were not improved. It is hypothetical that NER factors might have been lost this function during evolution.

Differently, data performed *in vitro* have shown that the mismatch repair (MMR) mechanism can target mismatches with rNMPs both in *E. coli* and *S. cerevisiae* genomic DNA (Shen, Koh, et al. 2011).

To date, there is no evidence that the BER mechanism could play any role in removing rNMPs from the genome. Increasing the knowledge in this field represents a stimulating goal in the DNA repair world and in the pathogenesis associated with this type of damage.

4.7 Beneficial effects of rNMPs incorporation

Mis-incorporation of ribonucleotides into DNA has been confirmed as a deleterious injury for the cell that, when not repaired, could destroy the genome integrity. A new vision has reinterpreted this phenomenon, hypothesizing a putative helpful and physiological effect of it for the cell.
Different are the evidences in support of this hypothesis.

In fission yeast, two rNMPs, consecutively incorporated in DNA, may mark the nascent DNA strand, initiating programmed mating-type switching (Sayrac et al. 2011).

Further, a recent work demonstrated that DNA polymerase mu (Pol μ), a member of the X-family of DNA polymerases operating in the repair of double strand breaks, possesses an open active site that does not allow a correct discrimination of the dNTPs (Brown et al. 2010; Ruiz et al. 2003). In other words, Pol μ is not able to discriminate the 2'-OH group of the sugar moiety and, for this reason, it will insert rNTPs as well as dNTPs during the NHEJ pathway (Martin et al. 2013). Although this could be considered detrimental for the cell, actually it might be advantageous. Inserting rNTPs with a higher base fidelity compared to dNTPs (Martin et al. 2013), Pol μ stimulates the DNA ligase IV and promotes the NHEJ mechanism (Nick McElhinny and Ramsden 2003).

Finally, another interesting hypothesis concerns a dual role of RNase H2. After the nicking at the 5’ side of rNTPs embedded in DNA by RNase H2, the remaining gap may enhance the efficiency of mismatch repair pathway. The MMR pathway, stimulated by the work of RNase H2, scans the DNA repairing eventual damages (Nick McElhinny et al. 2010; Lujan et al. 2013). Thus, the presence of rNTPs, when efficiently repaired by RNase H2, could stimulate the MMR initiation.

To date, data in support of putative beneficial effects of the rNMPs incorporation in DNA are few. Thus, further studies are ongoing in order to improve this open point.

4.8 Innovative tools for mapping rNMPs in DNA

An open point is understanding how the abundance and the distribution of ribonucleotides incorporated into DNA could change in different cells and under different stress conditions.

A step forward to go deeper on this topic, is represented by the development of different sophisticated approaches (Jinks-Robertson and Klein 2015), including Ribose-seq analysis (Koh, Balachander, et al. 2015), hydrolytic end-sequencing (HydEn-seq) analysis (Clausen et al. 2015), polymerase usage sequencing (PU-seq) analysis (Daigaku et al. 2015) and embedded ribose-sequencing (emRibo-seq) (Reijns et al. 2012).

All these approaches are able to efficiently tag the position of rNMPs within duplex DNA. They start with the processing of rNMPs embedded in DNA through NaOH treatment, that allows creating a basic environment promoting the alkaline cleavage of individual rNMPs present within DNA, thus generating fragments with terminal 2',3'-cycling phosphates or 2'-phosphates (PU-seq, HydEn-seq and Ribose-seq), or mediating RNase H2 cleavage at the 5' termini of rNMPs generating 3' OH termini (EmRibo-seq). Once isolated, these DNA products are isolated, amplified, and after that, sequenced with different sequencing approaches (Figure 15).

Use of these high-throughput tools has allowed obtaining more precise information about the distribution and abundance of rNMPs into the DNA.

From data obtained in yeast, it has been observed that rNMPs are incorporated into mitochondrial and nuclear DNA to a similar extent in a 'hotspot' manner (Koh, Balachander, et al. 2015). Moreover, in both nuclear and mtDNA, rCMP and rGMP are incorporated more frequently than the other nucleotides. Finally, the number of rNMPs identified per nuclear chromosome was found to be proportional to chromosome size (Koh, Balachander, et al. 2015). Quantitative approaches have estimated a 600 thousand rNMPs in budding yeast genome and over 100 million in mouse genome (Williams, Lujan, and Kunkel 2016).

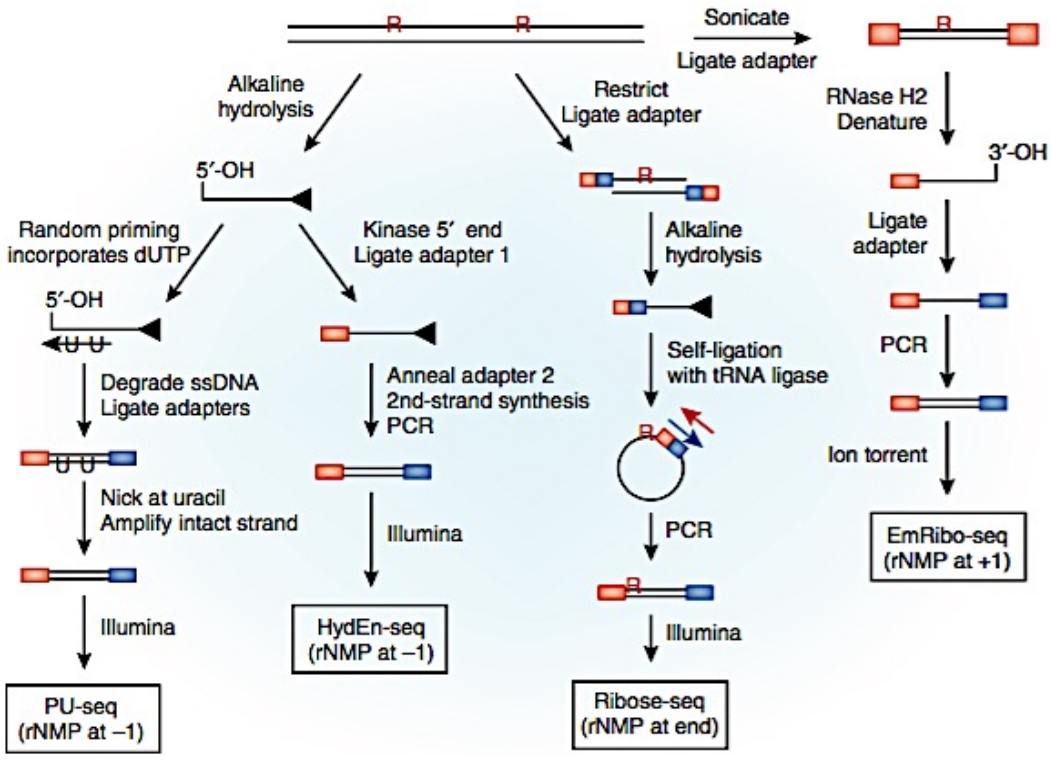


Figure 15 ***Summary of the four approaches developed to map rNMPs in DNA***

In PU-seq, HydEn-seq and ribose-seq, alkaline hydrolysis is used in order to isolate rNMPs-containing fragments that will be subsequently sequenced by Illumina methods. In EmRibo-seq, RNase H2 is used to cleave rNMPs and isolate the fragments with 3' OH termini that will be later sequenced by Ion torrent sequencing. (Jinks-Robertson and Klein, 2015)

5. Aim of this Study

The oxidation of biological molecules including nucleic acids is a very frequent phenomenon due, principally, to oxidative stress from endogenous and exogenous sources.

In particular, oxidation of RNA may have a fundamental significance for cell biology. For example, Zhan *et al.* have been reported that oxidized RNA compartmentalizes in cytoplasmic loci distinct from stress granules (Zhan et al. 2015). A recent quantitative analysis shows the presence of an atypical amount of oxidized RNA in Alzheimer's disease patients, highlighting a link between oxidized RNA and cell pathogenesis. Moreover, studies on investigating the biological effect of oxidatively and abasic damaged RNA demonstrated that these molecules interfere substantially with mRNA transcription (Kamiya et al. 2007) and ribosomal translation (Calabretta, Kupfer, and Leumann 2015). Although it was demonstrated that abasic RNA is processed by the BER pathway component, APE1 (Vascotto et al. 2009), data on the processing of oxidized RNA and the role of a specific glycosylase involved are missing.

Recently, the incorporation of ribonucleotides into the DNA has been better described as a new type of damage, caused during several physiological processes, such as replication (Williams, Lujan, and Kunkel 2016). The RER pathway represents the most important pathway involved in the removal of rNMPs embedded in DNA. Published data have demonstrated that well-known DNA repair pathways, including the MMR and the NER pathways, are not active when the RER does not work, as happens in some disorders.

To date, one open question is finding which DNA repair mechanisms are active as back-up when the RER does not work.

Moreover, it can be hypothesized that, among many millions rNMPs that are introduced in the mammalian genome per cell cycle (Williams, Lujan, and Kunkel 2016), damaged rNMPs (such as abasic and oxidized) can also be incorporated into the DNA. In fact, RNA molecules, as well as rNMPs present in the nucleotide pool, are also susceptible to oxidative insults (Randerath et

al. 1992; Moreira et al. 2008). The existence of abasic and oxidized rNMPs (such as 7,8-Dihydro-8-oxo-riboguanosine) in DNA and their repair represent an intriguing reason for study.

In this context, there is high likelihood that the BER pathway could be the best candidate in the processing of unmodified and modified rNMPs in DNA. The BER pathway works in the presence of non-bulky lesions generated by several damaging agents including oxidative stressors. Among BER enzymes, APE1 has a special role for all its multiple functions in cell biology and for its frequent involvement in different human pathologies ranging from neurodegenerative disorders to cancer (Li and Wilson 2014). Its role in the BER pathway is primarily due to its ability to act as 5' endonuclease, cleaving deoxy- abasic sites in DNA and ribo- abasic sites in RNA (Tell, Wilson, and Lee 2010; Vascotto et al. 2009; Berquist, McNeill, and Wilson 2008). But its involvement in the RNA world goes over, being able to have 3′-RNA phosphatase and a weak 3′-5′ exoribonuclease activities (Chohan et al. 2015). Moreover, APE1 is requested in pri-miRNA processing and stability via association with the DROSHA-processing complex during genotoxic stress (Antoniali et al. 2017). Finally, APE1 is involved in the nucleotide incision repair (NIR) pathway on modified bases, such as 5,6-dihydro-2'-deoxyuridine, 5,6-dihydrothymidine, 5-hydroxy-2'-deoxyuridine, 5-hydroxycytosine, in which, bypassing the action of specific glycosylases, directly cleaves the modified bases (Gros 2004b; Daviet et al. 2007b; Mazouzi et al. 2013).

Identifying whether the BER pathway, and especially APE1, may target normal and modified (abasic and oxidized) rNMPs in DNA represents a new amazing non-canonical role of this multifunctional protein that may help to better understand the mechanism of genotoxicity of reactive oxygen species as well as the function and the impact of BER defects in the molecular mechanism of different human diseases.

Thus, this Thesis has the purpose to show our data, obtained using sophisticated *in vitro* assay, in which different proteins belonging to the RER and the BER pathways were tested on unmodified and modified (abasic and oxidized) rNMPs embedded in DNA oligonucleotides, in order to discover the

existence of a DNA repair pathway specifically involved in the processing of these types of damage.

6. Materials and Methods

6.1 Synthetic oligonucleotides description and annealing conditions

All oligonucleotides used in this study are listed in Figure 16**a**.

Ribo- 1'OH abasic containing oligonucleotide, ss_rOH, were purchased from Dharmacon (GE Healthcare, Lafayette, CO, USA). The 26-mer oligonucleotide containing a tetrahydrofuran, ss_dF, and its reverse complementary sequence ss_dC, were synthesized from Metabion International AG (Steinkirchen, Germany). The 25-mer dG-, rG-, d8oxoG- containing oligonucleotides and complementary oligonucleotides were synthesized from Metabion International AG (Steinkirchen, Germany) (Figure 16**b**).

All oligonucleotides were labelled with either IRDye700, IRDye800 fluorophores or Cyanine5 at 5' end, purified through RP-HPLC, checked in Mass Check and re-suspended in RNase- and DNase- free water. Synthesis of oligonucleotide containing an internal ribose 8-oxo-guanosine (r8oxoG) and an IRDye700 fluorophore at 5' end was in-house carried out by our collaborator.

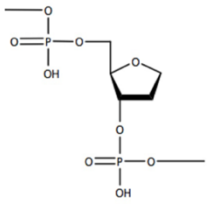
All oligonucleotides used in the present study were re-suspended in RNase- and DNase- free water at 100 µM. 100 pmol of each oligonucleotide was annealed with an excess of 150 pmol of its complementary DNA oligonucleotide in 10 mM TrisHCl pH 7.4 and 10 mM $MgCl_2$, heated at 95°C and cooling down over night in the dark.

a)

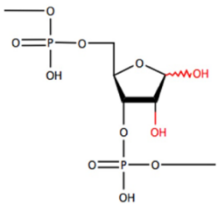
	Name	Sequence (5' → 3')	Complementary strand(3' → 5')
DNA oligo control	ds_dG:dC	GGATCCGGTAGTdGTTAGGCCTGAAC	CCTAGGCCATCACAATCCGGACTTG
	ds_d8oxoG:dC	GGATCCGGTAGT^{8oxo}dGTTAGGCCTGAAC	CCTAGGCCATCACAATCCGGACTTG
	ds_d8oxoG:dA	GGATCCGGTAGT^{8oxo}dGTTAGGCCTGAAC	CCTAGGCCATCAAAATCCGGACTTG
	ds_dF:dC	AATTCACCGGTACCd^{AP}TCTAGAATTCG	CGAATTCTAGAGGGTACCGGTGAATT
unmodified rNMP in DNA oligo	ds_rG:dC	GGATCCGGTAGTrGTTAGGCCTGAAC	CCTAGGCCATCACAATCCGGACTTG
	ds_rG:dA	GGATCCGGTAGTrGTTAGGCCTGAAC	CCTAGGCCATCAAAATCCGGACTTG
modified rNMP in DNA oligo	ds_r8oxoG:dC	GGATCCGGTAGT^{8oxo}rGTTAGGCCTGAAC	CCTAGGCCATCACAATCCGGACTTG
	ds_r8oxoG:dA	GGATCCGGTAGT^{8oxo}rGTTAGGCCTGAAC	CCTAGGCCATCAAAATCCGGACTTG
	ds_rOH:dC	GGATCCGGTAGTr^{AP}TTAGGCCTGAAC	CCTAGGCCATCACAATCCGGACTTG

b)

Deoxyribose tetrahydrofuran
(dF modification)



Ribose abasic site
(rOH modification)



Ribose 8-Oxo-7,8-dihydro-guanosine
(r8oxoG modification)

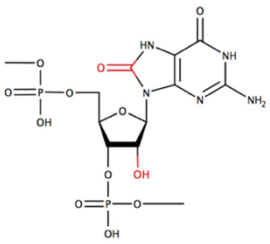


Figure 16 ***View of the modified oligonucleotides used in the current study***

a) *Oligonucleotides list, organized for three subtypes that are: DNA oligo control, unmodified rNMP in DNA oligo and modified rNMP in DNA oligo. In the table, all the single-stranded oligonucleotides sequences and their complementary sequences are specified. Unmodified and modified deoxyribonucleotides are colored in blue whereas unmodified and modified ribonucleotides in red.* ***b)*** *Chemical representation of the deoxy- tetrahydrofuran, ribose 1'OH abasic site and ribose 8-Oxo-7,8-dihydro-guanosine modifications.*

The r8oxoG synthesis was carried out on an Applied Biosystems 392 DNA/RNA synthesizer using the phosphoramidite chemistry, associated with the phenoxyacetyl protecting group for the nucleobases and the *tertio*-butyldimethylsilyle protecting group at the 2'-OH position of the ribonucleoside residue (Gasparutto et al. 1992). Upon completion, the oligonucleotide was de-protected in concentrated aqueous ammonia for 6 hours at 55°C, followed by a desilylation step with triethylamine trihydrofluoride (8 hours at room temperature) (Gasparutto et al. 1992) and was finally purified by preparative 20% denaturing PAGE using UV-shadowing detection. After desalting by size exclusion, the r8oxoG oligonucleotide was quantified by UV measurements at 260 nm and its purity was checked by RP-HPLC analysis together with MALDI-TOF mass measurements (Figure 17). Sample was then lyophilized and frozen at -20°C until use.

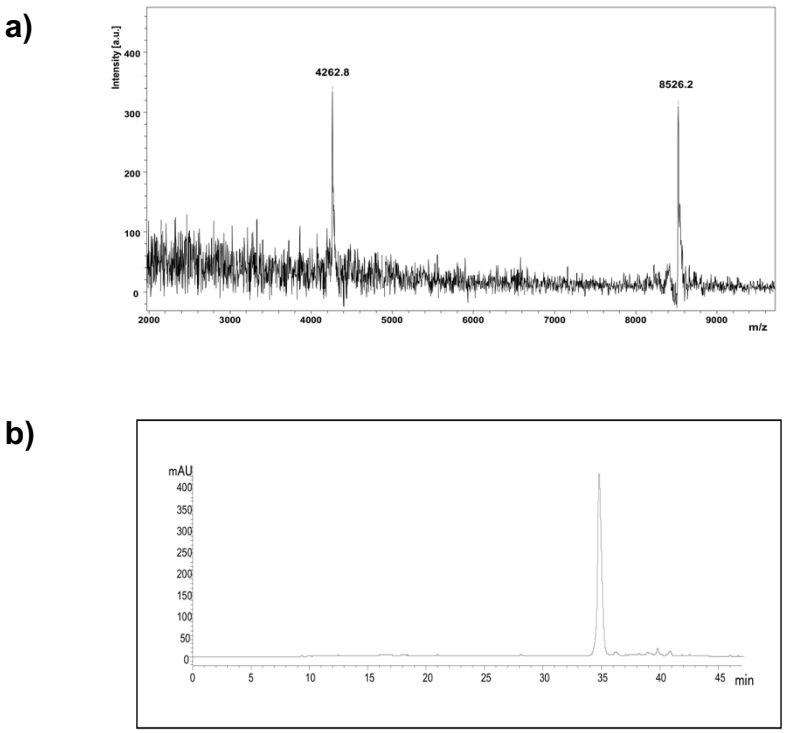


Figure 17 ***r8oxoG- containing DNA oligonucleotide analysis***

a) *MALDI-TOF mass spectrum of the r8oxoG- containing DNA oligonucleotide (calculated mass: 8528.9; measured mass: 8527.2) shows an unique fragment with two states of charge: Peak [M-H]- : 8526.2 and Peak [M-2H]2- : 4262.8.* ***b)*** *Reversed-phase chromatography of the labeled modified oligonucleotide (Gradient of 0 to 35% of CH3CN in TEAA 10mM in 45min – detection 254nm). (Courtesy of Dr. Gasparutto- published in Malfatti et al., 2017)*

6.2 Plasmid and expression of recombinant proteins

Plasmids and expression of human recombinant OGG1 enzyme was purified as described in Audebert *et al*., (Audebert, Radicella, and Dizdaroglu 2000). Plasmids and expression of human recombinant APE1 wild type (WT) and respective mutants (APE1 NΔ33 and APE1 E96A) were produced as explained in Fantini *et al*., (Fantini et al. 2010) and in Erzberger and Wilson (Erzberger and Wilson 1999). Plasmid and expression of yeast, mouse and human recombinant RNase H2 were produced as explained in Chon *et al.*, (Chon et al. 2009; Hyongi Chon et al. 2013).

6.3 Cell lines and silencing experiments

HeLa cells (human cervical carcinoma) (ATCC®, Milan, Italy) were grown in DMEM (EuroClone, Milan, Italy) supplemented with 10% fetal bovin serum (FBS-EuroClone, Milan, Italy), penicillin (100 U/ml), streptomycin (100 mg/ml) and l-glutamine (2 mM) (EuroClone, Milan, Italy) and cultured in a humidified incubator at 5% CO_2 at 37°C. For silencing experiments, 15 × 10^4 cells were seeded and transfected with 5' UACUCCAGUCGUACCAGACCU 3' siAPE1 (100 pmol) or siGENOME SMART pool siRNase H2A (50 pmol) or 5’ CCA UGA GGU CAG CAU GGU CUG UU 3’ scramble control siRNA (100 pmol) (GE Dharmacon, Milan, Italy) by using Oligofectamine™ Reagent (GE Dharmacon, Milan, Italy) as per manufacturer’s indications. After 72 hours upon transfection, cells were harvested by trypsinization and centrifuged at 250 × g for 5 minutes at 4°C. Supernatant was removed, and pellet was washed once with ice-cold phosphate-buffered saline without Calcium and Magnesium (PBS-Euroclone, Milan, Italy) and then centrifuged again (250 × g for 5 minutes at 4°C).

6.4 Preparation of nuclear cell extracts (NCE)

After washing with PBS, cells were collected in cold PBS added with 0.1 M DTT and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were centrifuged at 800 × g for 10 minutes at 4°C and the supernatant was removed. Pellet was re-suspended in a cold hypotonic solution containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA pH 8.0 complemented with 0.1 mM DTT, 0.5 mM PMSF, 1 mM protease inhibitor (PI), 1 mM NaF, 1 mM Na_3VO_4.

After centrifugation at 800 × g for 10 minutes at 4°C, cytosolic proteins (CCE) were collected whereas intact nuclei were pelleted. Pellet was washed to discard any contamination from cytosol and it was subsequently re-suspended with a cold hypertonic solution 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA pH 8.0, 5% glycerol complemented with 0.1 mM DTT, 0.5 mM PMSF, 1 mM PI, 1 mM NaF, 1 mM Na_3VO_4 and incubated on ice for 30 minutes. At the end, the sample was centrifuged at 15,000 × g for 20 minutes at 4°C and collected the supernatant containing nuclear proteins (NCE). Quantification of each sample was performed by colorimetric Bradford assays (Bio-Rad, Milan, Italy).

6.5 Preparation of whole cell extracts (WCE)

After washing with PBS, cells were harvested by trypsinization and centrifuged at 250 × g for 5 minutes at 4°C. The supernatant was removed, washed once with PBS and centrifuged again. Pellet was re-suspended in a lysis solution containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% wt/vol Triton X-100 supplemented with 1 mM PI, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4. After centrifugation at 15,000 × g for 20 minutes at 4°C, the supernatant is considered as whole cell extract (WCE). Proteins of each sample were quantified using a colorimetric Bradford assays (Bio-Rad, Milan, Italy).

6.6 SDS-PAGE electrophoresis separation, detection with Coomassie staining and Western blot analysis of protein samples

All recombinant proteins were loaded onto a 10 w/vol % sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) electrophoresis gel, which was subsequently stained using Coomassie Brilliant Blue stain (ThermoFisher, Waltham, MA, USA). Each band, corresponding to the protein of interest, was quantified and normalized with a BSA (bovine serum albumin) standardization curve. The image was finally developed by using NIR Fluorescence technology with an Odissey CLx scanner (LI-COR GmbH, Germany). Bands were quantified and analyzed using the ImageStudio software (LI-COR GmbH, Germany).

Cell extracts samples were loaded onto a 12 w/vol % SDS-PAGE electrophoresis gel. Proteins were then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Monoclonal α-APE1 was from Novus Biologicals (Littleton, CO, USA-NB 100-116), polyclonal α-RNase H2 was from Abcam (Cambridge, UK - ab92876). Monoclonal α-tubulin from SIGMA-ALDRICH (Milan, Italy - T-9026) and α-Lamin A from Abcam (Cambridge, UK - ab8980) antibodies were used to detect the respective proteins (i.e. Tubulin and Lamin A) as protein normalizers for whole/cytoplasmic and nuclear cell extracts respectively. Membranes were incubated with secondary antibodies labeled with IRDye (1:10,000 dilution) in 5% milk, PBS and Tween 0.1% and finally developed by using NIR Fluorescence technology with an Odissey CLx scanner (LI-COR GmbH, Germany). Bands were quantified and analyzed using the ImageStudio software (LI-COR GmbH, Germany).

6.7 Endonuclease assay

To measure enzymatic activity of recombinant proteins and NCE on different substrates, each reaction was prepared following doses, time points and buffers specified in detail into the legend of each experiment. Final volume for each reaction was 10 µl. At the end of all reactions, samples were blocked with a stop solution, containing 99.5% v/v Formamide (SIGMA-ALDRICH, Milan, Italy) supplemented with 10X Orange Loading Dye (LI-COR Biosciences, Milan, Italy) and heated at 95°C for 5 minutes. Then, all samples were loaded onto a 7 M denaturing 20% polyacrylamide gel in TBE buffer pH 8.0 and run at 4°C at 300V for 1 hour. Then, the gel was visualized with an Odyssey CLx Infrared Imaging system (LI-COR GmbH, Germany). The signals of the non-incised substrate (S) and the incision product (P) bands were quantified using Image Studio software (LI-COR GmbH, Germany). In all the experiments, we indicated with '*Free*' term, the oligonucleotide incubated without any protein or cell extract. In each relative graph, we plotted the percentage of endonuclease activity on *y* axis, calculated as the quote of product divided for the total of oligonucleotide used. When using the ds_rOH:dC and ds_r8oxoG:dC oligonucleotides, a very small amount of cleavage product was seen in samples not treated with recombinant proteins

and/or cell extracts due to the reactivity of this molecule, which was spontaneously degraded. During the analysis, this band has been always subtracted from bands obtained following treatment with recombinant proteins and/or extracts (Figure 18).

6.8 Electrophoretic mobility shift assay analysis (EMSA)

Proteins binding to nucleic acids was assessed by EMSA analysis as already described by Fantini et al., (Fantini et al. 2010). Briefly, the indicated amounts of recombinant purified proteins or cell extracts were co-incubated with 250 fmol of the probe (25 nM) at 37°C for 30 minutes. Reactions were prepared in a buffer containing 8 mM HEPES, 10 mM KCl, 400 µM EDTA pH 8.0, 5 mM DTT and 2% glycerol in a 10 µl final volume. Moreover, salmon sperm DNA (SSD) (SIGMA-ALDRICH, Milan, Italy) was added like as DNA competitor. Samples were loaded onto an 8% w/vol native polyacrylamide gel in Tris-Sodium Acetate-EDTA pH 8.0 (TAE) buffer and run at 4°C at 150V for 1 hour followed by 3 hours at 250V (Figure 18). Specific proteins belonging to the protein complex-DNA were detected using a supershift EMSA analysis that couples an EMSA analysis, already explained in Material and Methods, to an additional 1 hour of pre-incubation of monoclonal antibody direct versus APE1 (Novus Biologicals, Littleton, CO, USA-NB 100-116) with the total cell extract at 4°C. In all the experiments, we indicated with '*Free*' term, the oligonucleotide incubated without any protein or cell extract.

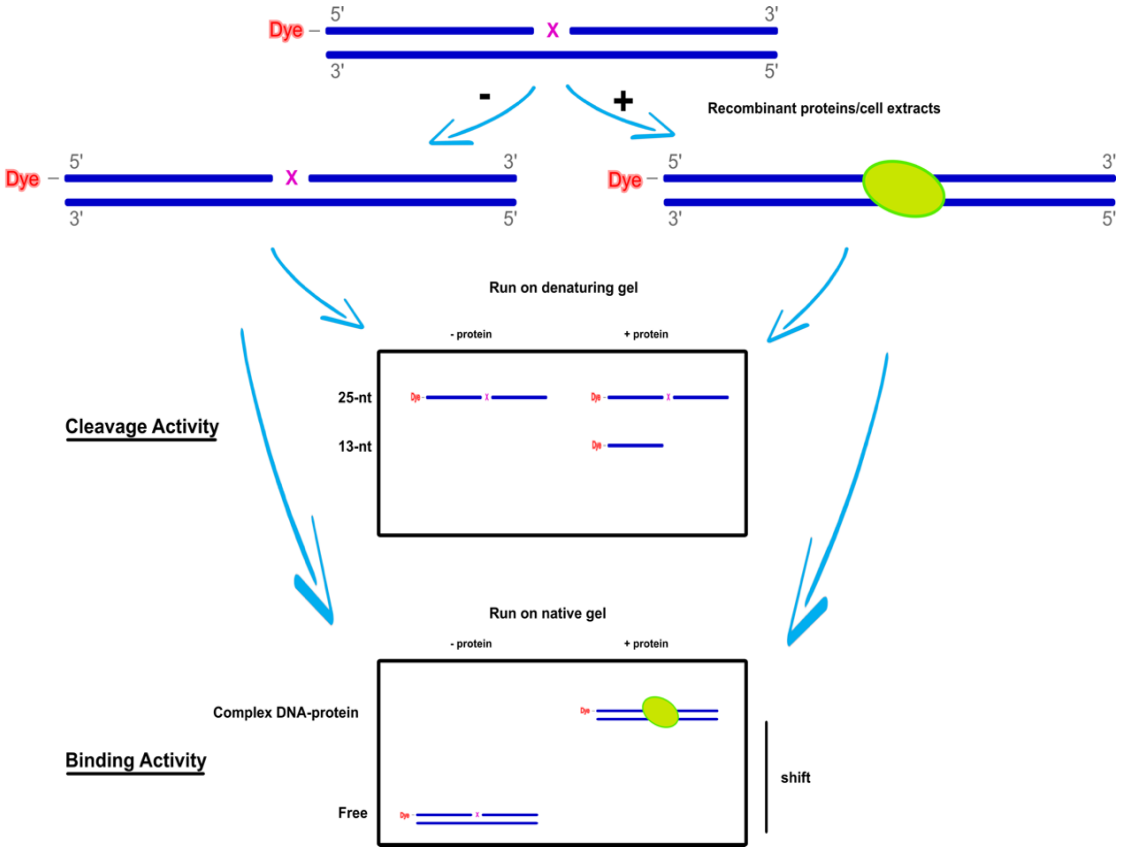


Figure 18 ***Schematic representation of enzymatic assays***

Upon the reaction between oligonucleotides and proteins is occurred in specific buffer, time and doses conditions, the reaction is run or on a denaturing gel in order to observe a cleavage activity of the protein on the oligonucleotide, or on a native gel in order to discriminate a shift of the signal due to a binding between protein and oligonucleotide.

6.9 Data representation and statistical analysis

All graphs were executed and statistically analyzed by using *GraphPad* software. Statistical analyses were performed by using the Student's t test. $P<0.05$ and $P<0.001$ were considered as statistically significant.

7. Results

7.1 Human RNase H2 does not process a rAP site embedded in DNA

RNase H2 is the principal protein able to process paired and mismatched rNMP sites embedded in DNA by generating a nick of their 5′ side (Shen, Koh, et al. 2011). To date, whether RNase H2 can cleave a rAP site incorporated in a duplex DNA is unknown.

In order to test this hypothesis, we measured RNase H2 ability to cleave a modified 25-mer DNA oligonucleotide, called ds_rOH:dC, in which a 1'-OH abasic rNMP was incorporated into a DNA substrate as shown in Figure 16 and Figure 19. In parallel other three oligonucleotides were used including a dG- containing oligonucleotide called ds_dG:dC, as negative control, rG-containing oligonucleotides paired with C or A in the complementary strands, called ds_rG:dC and ds_rG:dA respectively as positive control, and finally the corresponding abasic modification with a deoxyribose instead of a ribose sugar, called ds_dF:dC (Figure 16 and Figure 19).

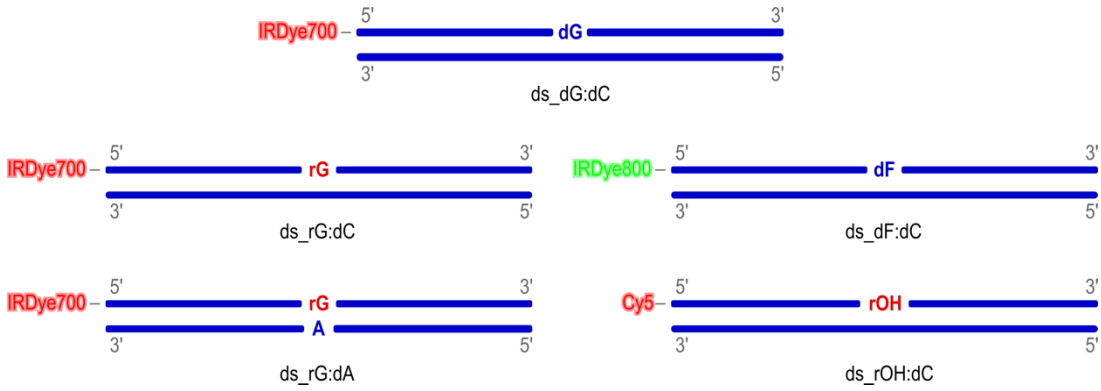


Figure 19 ***Scheme of substrates used to test the processing of a rAP site embedded in a duplex DNA substrate***

Double-stranded (ds) DNA substrates containing a dGMP, rGMP, dF and 1' OH abasic rNMP (rOH) sites in the 13th position. The 'IRDye700' in red indicates IRDye 700 phosphoramidite dye tagged at the 5' end. The 'IRDye800' in green indicates IRDye 800 phosphoramidite dye tagged at the 5' end. The 'Cy5' in red indicates cyanine dye tagged at the 5'-end of the top strand of the duplex. DNA nucleotides are in blue, RNA in red. The 5' and 3' ends of each DNA strand are indicated.

First of all, recombinant human RNase H2, composed of its three subunits, was purified as explained in Materials and Methods (Figure 20) and its activity was tested on ds_rOH:dC oligonucleotide in parallel with dG- and rG- containing oligonucleotides as negative and positive controls, respectively.

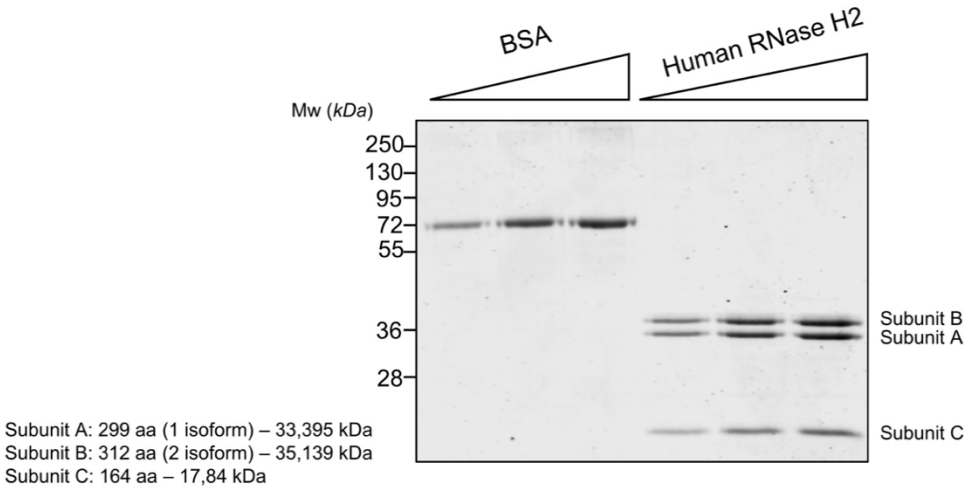


Figure 20 ***Gel-quantification of human RNase H2 recombinant protein used in this study***

Increasing doses (250-500-750 ng) of human recombinant RNase H2 protein was separated onto 10% SDS–PAGE gel followed by Coomassie staining. Bands corresponding to each protein were quantified and normalized on a standardization curve of Bovine Serum Albumin (BSA) protein (250-500-750 ng). The molecular weight (Mw) expressed in kilodaltons (kDa) is shown on the right of each panel.

As reported in Figure 21, the enzyme had no activity on ds_dG:dC and ds_dF:dC (containing a tetrahydrofuran residue mimicking the abasic site) oligonucleotides, whereas it efficiently cleaved the canonical rG substrate as expected. In addition, we tested the mismatched rG- containing oligonucleotide, ds_rG:dA, confirming that RNase H2 protein is able to cleave rGMP in mismatch with dAMP.

On the other hand, no activity was detectable on ds_rOH:dC demonstrating that recombinant human RNase H2 is not able to process an abasic rNMP embedded in DNA.

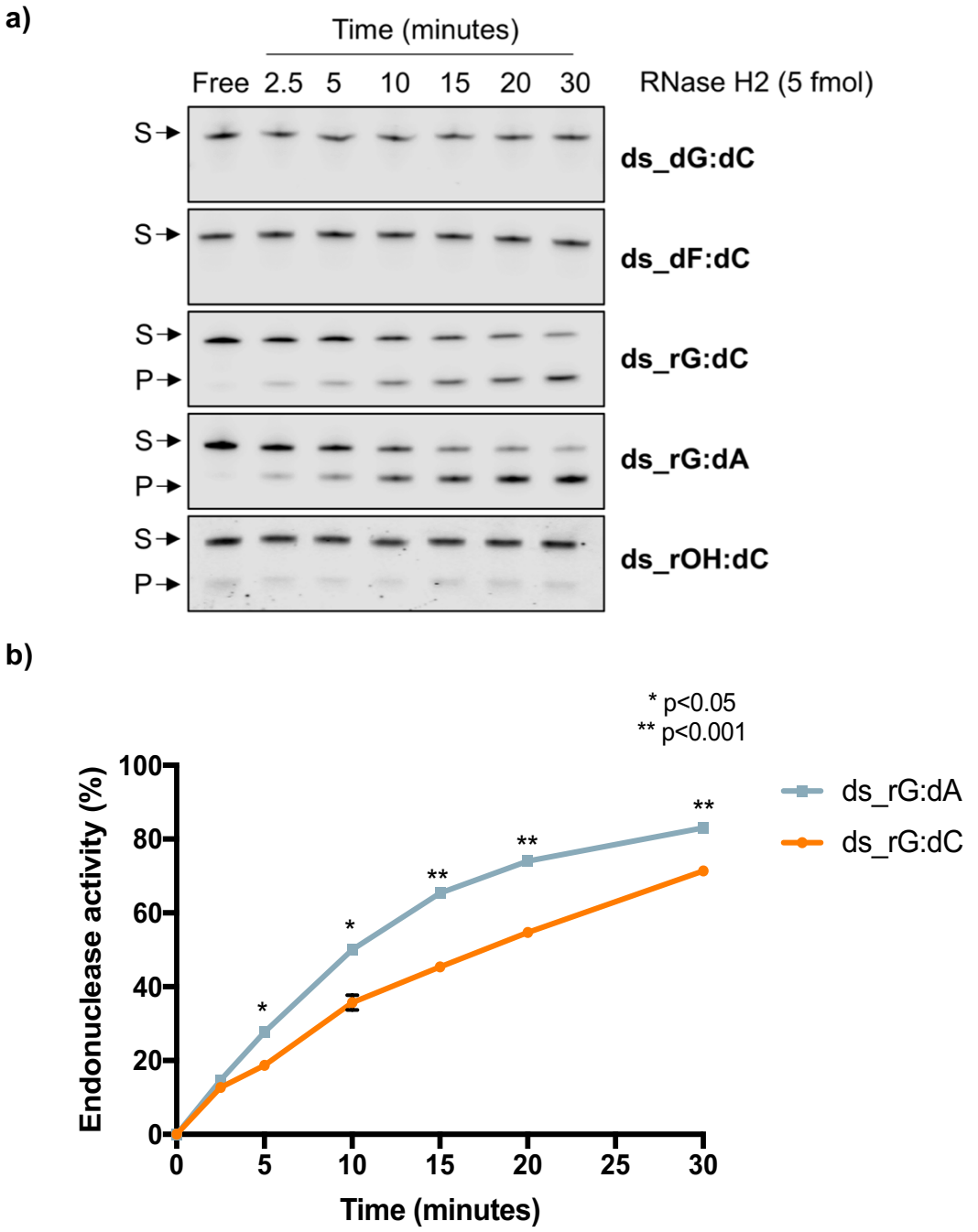


Figure 21 ***Human recombinant RNase H2 protein is not able to process an rAP site embedded in a duplex DNA substrate***

a) *Representative denaturing polyacrylamide gel of oligonucleotides (25 nM) incision by recombinant human RNase H2 (0.5 nM). The reaction was performed in RNase H2-buffer (20 mM Tris-HCl, 25 mM KCl, 0.1% BSA, 0.01% Tween20, 4 mM $MgCl_2$, pH 7.4) for different time points, expressed in minutes and shown on the top of the figure, at 37°C. ds_dG:dC and ds_dF:dC oligonucleotides were used as negative controls whereas paired and mismatched ds_rG oligonucleotides as positive controls. S indicates the substrate position while P indicates the product position.* ***b)*** *Relative graph illustrating the time-course kinetics activity of the recombinant protein on ds_rG:dC and ds_rG:dA oligonucleotides. Data are expressed as mean ± SD of three independent technical replicas. Statistical significant differences, calculated through t-Test analysis, are indicated as *p<0.05 and **p<0.001.*

Later, we proceeded to confirm these data *in vivo* using cell extracts. We tested the activity of RNase H2 protein obtained from nuclear cell extracts on the same substrates, as above. To this aim, RNase H2 expression was downregulated in HeLa cells through specific siRNA and the endoribonuclease activities of nuclear extracts from knocked down and control cells were then assayed. Western blotting analyses, performed on nuclear extracts from control (Scramble) and knocked down (siRNase H2) cells, demonstrated the efficiency of RNase H2 downregulation (about 50%) upon transfection with specific siRNA sequences (Figure 22). APE1 silencing and double APE1 + RNase H2 silencing, shown in the WB panel below, will be explained later.

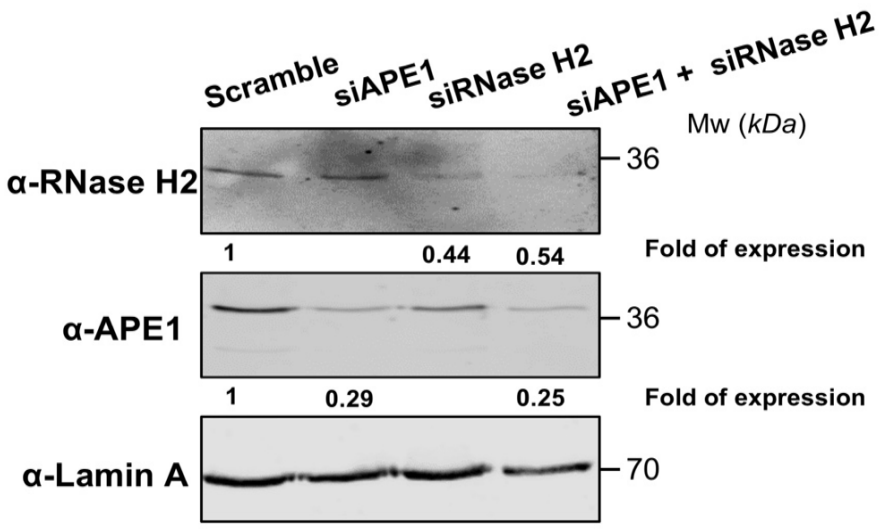


Figure 22 ***Western blot silencing analysis on nuclear HeLa cell extracts***

HeLa cells were transfected with specific siRNAs directed versus RNase H2 and APE1 proteins and compared to the Scramble control. Nuclear cell extracts were separated onto 12% SDS–PAGE, and Western blot analysis was performed by using an anti-APE1 antibody and an anti-RNase H2 antibody. Lamin A was used as loading control. Expression levels for each condition was normalized to the Scramble and indicated under each corresponding lane. The molecular weight (Mw) expressed in kilodaltons (kDa) is shown on the right of each panel.

We incubated Scramble or siRNase H2 cell extracts with different substrates for the indicated time points. Following knock-down of RNase H2, we found a decreased cleavage of ds_rG:dC, as expected Figure 23. Surprisingly, we

found that ds_rOH:dC was also cleaved; however, ds_rOH:dC cleavage was completely unaffected by siRNase H2 cell extracts.

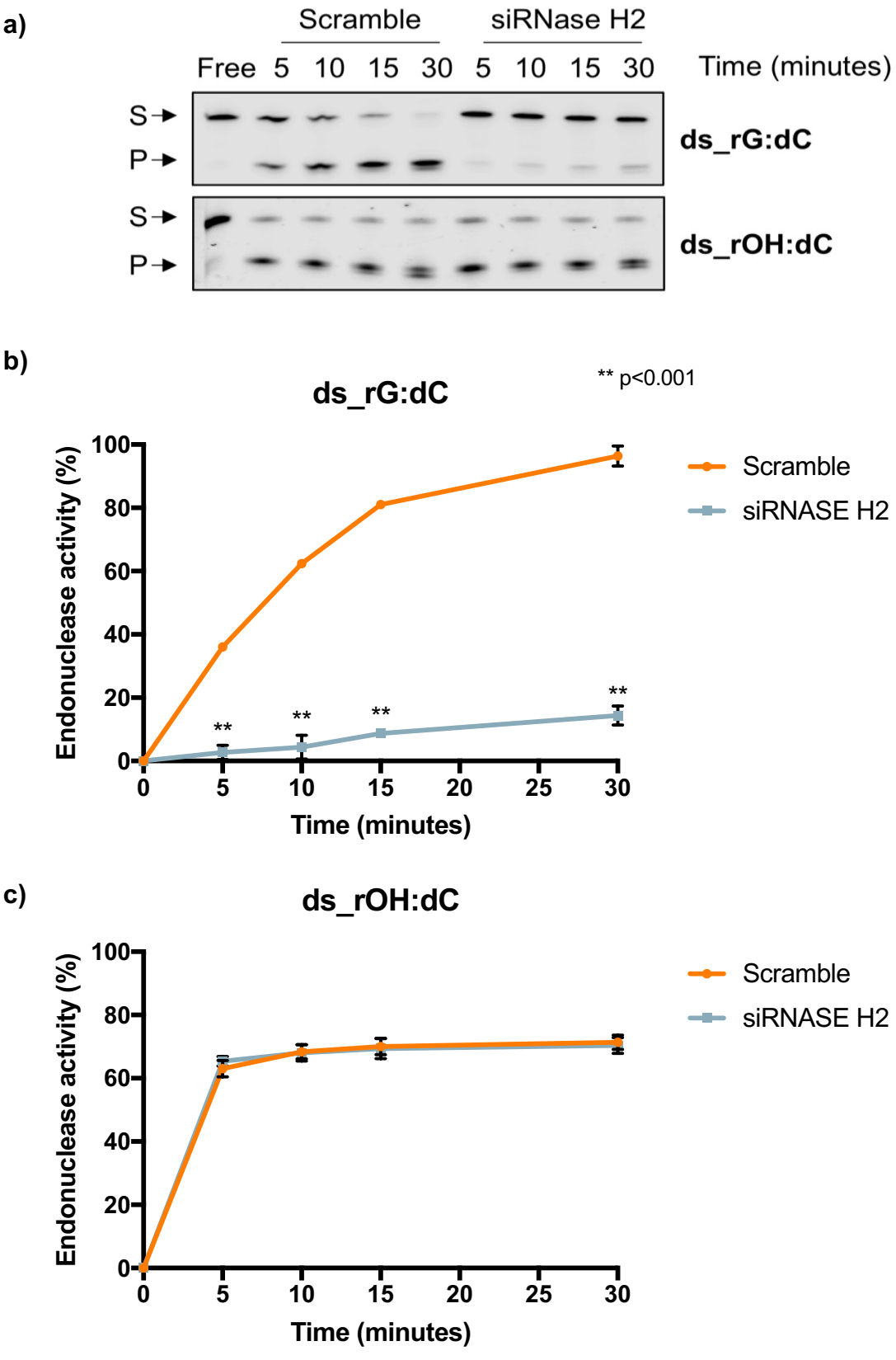
These data suggest that human RNase H2 is inactive on an abasic rNMP embedded in DNA, hypothesizing that there may be another enzyme/s capable of cleaving it.

In addition from *data not shown*, in order to increase the stability of the abasic rNMP-containing oligonucleotide, an abasic substrate mimicked by tetrahydrofuran (F) residues, similarly to what commonly used for dNMP (Wilson 2005; Poletto, Malfatti, et al. 2016) was used. Moreover, a longer DNA sequence was chosen (about 40-bp) in order to evaluate a possible role of the length of the substrate in determining the inability of RNase H2 to process these substrates. Recombinant RNase H2 proteins from yeast *S. cerevisiae* or mouse were tested on these different substrates and, as expected and confirming the previous data, both proteins were unable to cleave abasic rG sites in DNA (Malfatti et al. 2017).

Together, these results demonstrate and confirm that eukaryotic RNase H2, either from yeast or mammalian origins, do not process abasic rNMP incorporated in DNA, independently from the nature of the abasic site (either 1'-OH or tetrahydrofuran residue) and the length of the substrate (either 25- or 40-mers).

Figure 23 ***RNase H2 from cell extracts is unable to cleave rAP sites in DNA***

a) *Representative denaturing polyacrylamide gel of oligonucleotides (0.25 μM) incision by nuclear HeLa cell extracts (NCE). In order to discriminate the activity of RNase H2, 500 ng of NCE, in which RNase H2 expression was previously knocked down through specific siRNA (indicated as siRNase H2), were tested in comparison to control cells (Scramble) at different time points, expressed in minutes. rG-containing oligonucleotide was used as positive control. Time points, expressed in minutes, are shown on the top of the figure. Enzymatic reaction was performed at 37°C in RNase H2-buffer. S indicates the substrate position, while P indicates the product position.* ***b)*** *Graph illustrating the time-course kinetics activity of NCE on ds_rG:dC in control and RNase H2-knocked down conditions. Enzymatic reaction was performed at 37°C in RNase H2-buffer with 500 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always less than 10% of the mean of the experimental points. Statistical significant differences, calculated through t-Test analysis, are indicated as *p<0.05 and **p<0.001.* ***c)*** *Graph illustrating the time-course kinetics activity of NCE on ds_rOH:dC oligonucleotide in control and RNase H2-knocked down conditions. Enzymatic reaction was performed at 37°C in RNase H2-buffer with 500 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always less than 10% of the mean of the experimental points.*



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7.2 Human APE1 is able to process a rAP site embedded in DNA through its endonuclease catalytic domain

Upon demonstration that RNase H2 enzyme was inefficient in the processing of abasic ribonucleotides embedded in DNA, we moved our attention on APE1, the AP endonuclease deputed on cleave abasic sites in DNA during the BER (Tell et al. 2009).

Therefore, in order to test the ability of APE1 to process rAP sites in DNA, we purified human recombinant APE1 protein, as described in Materials and Methods section (Figure 24).

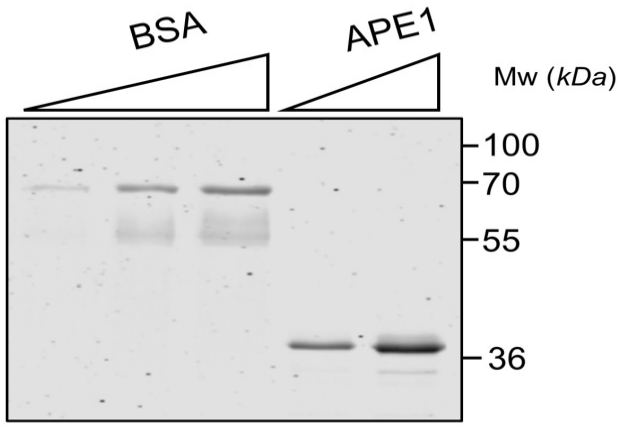


Figure 24 ***Gel-quantification of recombinant APE1 WT protein used in this study***

Increasing doses (500-750 ng) of human recombinant APE1 WT protein was separated onto 10% SDS–PAGE gel followed by Coomassie staining. Bands corresponding to each protein were quantified and normalized on a standardization curve of Bovine Serum Albumin (BSA) protein (250-500-750 ng). The molecular weight (Mw) expressed in kilodaltons (kDa) is shown on the right of each panel.

The endonuclease activity of APE1 on ds_rOH:dC substrate was examined through cleavage assays. As a positive control for APE1 endonuclease activity, an oligonucleotide substrate containing a tetrahydrofuran residue mimicking the abasic site, called ds_dF:dC (Wilson 2005; Poletto, Malfatti, et al. 2016), was used (Figure 16 and Figure 19). As reported in Figure 25, and measured through kinetics experiments in

Table *1*, APE1 processes the abasic rNMP within DNA as efficiently as the canonical abasic dNMP.

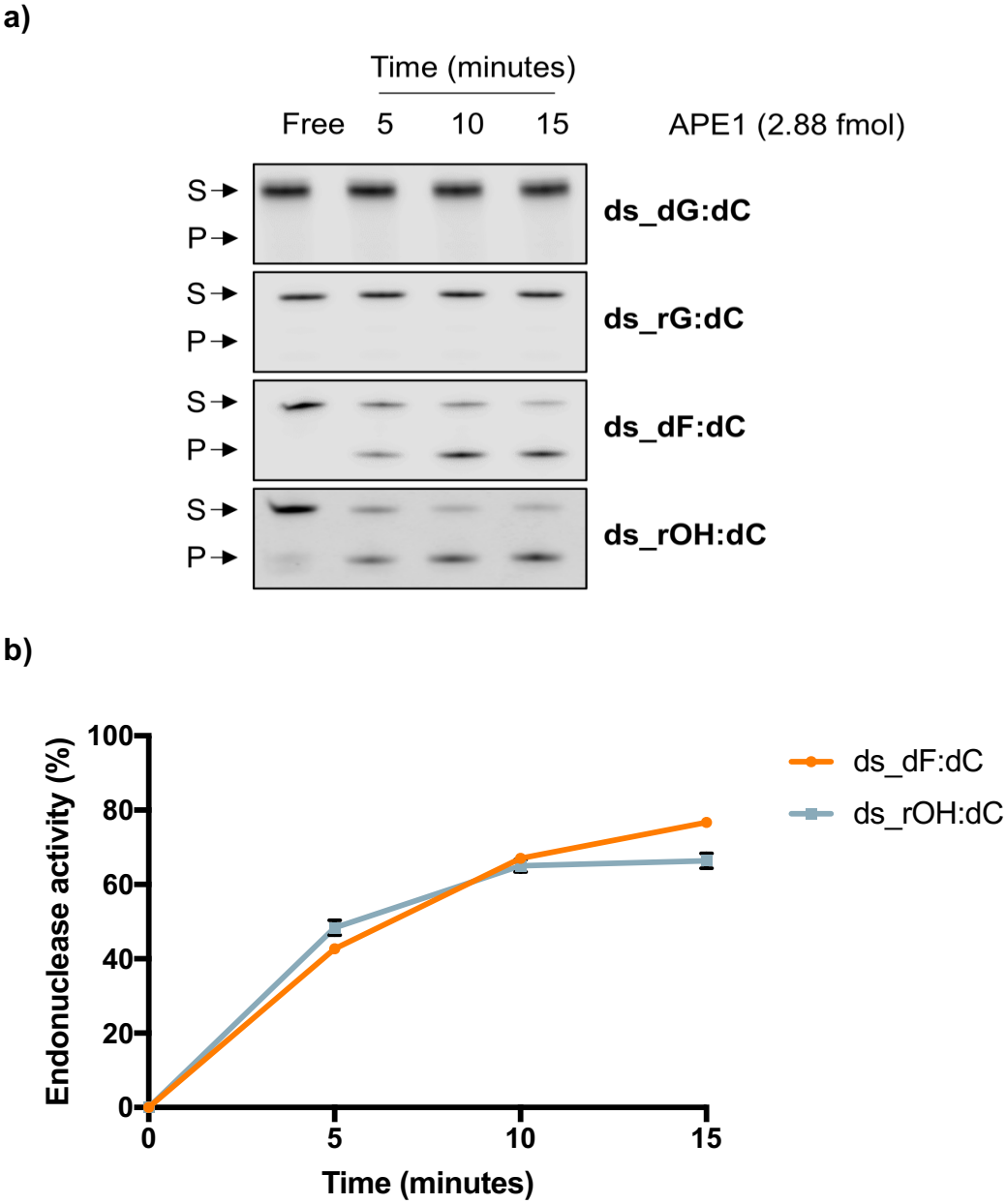


Figure 25 ***Recombinant human APE1 protein efficiently processes a rAP site embedded in DNA***

a) *Representative denaturing polyacrylamide gel of oligonucleotides (0.25 μM) incision by recombinant APE1 protein (0.288 nM). The reaction was performed in APE1-buffer for different time points, expressed in minutes and shown on the top of the figure, at 37°C. ds_dF:dC oligonucleotide was used as positive control. S indicates the substrate position while P indicates the product position.* ***b)*** *Relative graph illustrating the time-course kinetics activity of the recombinant protein on ds_dF:dC and ds_rOH:dC oligonucleotides. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always <10% of the mean of the experimental points*

Moreover, we clearly found that APE1 was unable to process the rG-containing oligonucleotide, which is the preferential substrate of RNase H2 enzyme (Figure 25), while it is active on the rOH substrate.

We then measured the specific activity of APE1 on abasic ribonucleotide embedded in DNA, through kinetis experiments. The kinetic constants were calculated and are reported in

Table 1. Comparing the values, we observe that APE1 has a lower affinity for the ds_rOH:dC than the ds_dF:dC (11-fold increase of the K_M) but a higher catalytic rate (27-fold increase in the k_{cat}/K_M ratio).

[APE1] ($\times 10^{-3}$ nM)	Substrate	K_M [nM]	V_{MAX} (nM/min)	k_{CAT} (min^{-1})	k_{CAT}/K_M $(min*nM)^{-1}$
75	ds_dF:dC	14.2±6.98	0.95±0.33	12.7±4.39	0.95±0.16
3.125	ds_rOH:dC	158±41.79	12.66±3.71	4054±1191.8	26±1.96

Table 1 ***Kinetic parameters for APE1 endonuclease activity on different substrates***

Kinetic parameters (K_M, V_{MAX} and k_{CAT}) were calculated from the measurement of the endonucleolytic reaction rates for APE1 on ds_dF:dC and ds_rOH:dC substrates. As described in Fantini et al., increasing concentrations of the substrate were incubated with a selecting concentration of the protein (see first column) in a time-course experiment. Kinetic values were calculated using a Lineweaver-Burk plot analysis and represent the mean ± SD of three independent experiments.

In order to further characterize the enzymatic activity of APE1, we used the purified recombinant mutant APE1 E96A protein, in which an E→A aminoacid substitution was inserted into the catalytic site. The aminoacid substitution causes a decreased enzymatic activity of the protein, due to the inability to coordinate the Mg^{2+} ion in the catalytic site (Wilson 2005; Beernink et al. 2001; Izumi et al. 2005b) (Figure 26). In addition, we also used the purified recombinant mutant APE1 NΔ33 protein, in which the first 33 N-terminal residues, responsible for RNA-protein interaction but not affecting its enzymatic activity, have been deleted (Lindahl 1993; Tell, Wilson, and Lee 2010) (Figure 26).

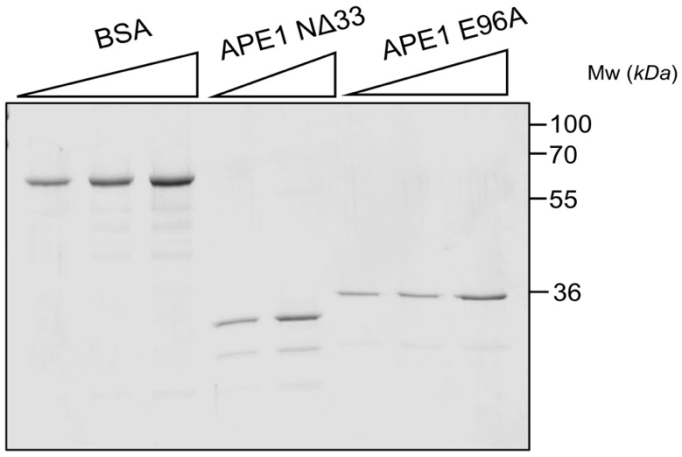


Figure 26 ***Gel-quantification of mutant APE1 recombinant proteins***

Human recombinant mutants APE1 NΔ33 (250-500 ng) and APE1 E96A (250-500-750 ng) proteins were separated onto 10% SDS–PAGE gel followed by Coomassie staining. Bands corresponding to each protein were quantified and normalized on a standardization curve of Bovine Serum Albumin (BSA) protein (250-500-750 ng). The molecular weight (Mw) expressed in kilodaltons (kDa) is shown on the right of each panel.

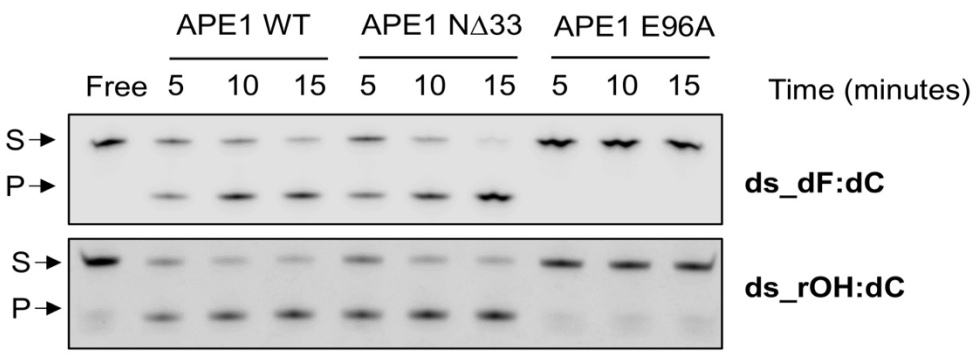
Following incubation of ds_rOH:dC with APE1 E96A mutant, there was barely any endonuclease activity, whereas the activity of APE1 NΔ33 mutant was comparable with that of the APE1 WT protein (Figure 27).

These data demonstrate that the catalytic domain of APE1 is responsible for recognizing and cleaving a rAP site in dsDNA and that the N-terminal domain does not play any major role in the enzymatic activity on this substrate and that AP endonucleolytic activity on rAP sites is intrinsic to the purified protein.

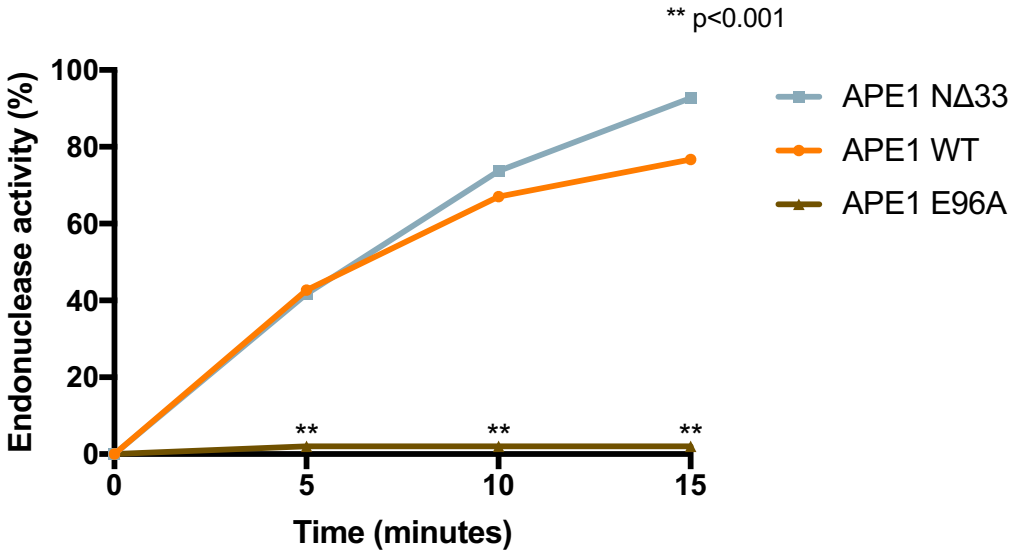
Figure 27 ***AP catalytic domain of APE1 is involved in the cleavage of abasic rNMPs embedded in DNA***

a) *Representative denaturing polyacrylamide gel of oligonucleotides (2.5 pmol) incision by different APE1 mutants NΔ33 and E96A (0.288 nM), in comparison to wild type APE1 (WT). The reaction was performed in APE1-buffer for different time points, expressed in minutes and shown on the top of the figure, at 37°C. ds_dF:dC oligonucleotide was used as positive control. S indicates the substrate position while P indicates the product position.* ***b)*** *and* ***c)*** *Graphs illustrating the time-course kinetics activity of APE1 mutants on ds_dF:dC and ds_rOH:dC oligonucleotides. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always <10% of the mean of the experimental points. Statistical significant differences, calculated through t-Test analysis, are indicated as **p<0.001.*

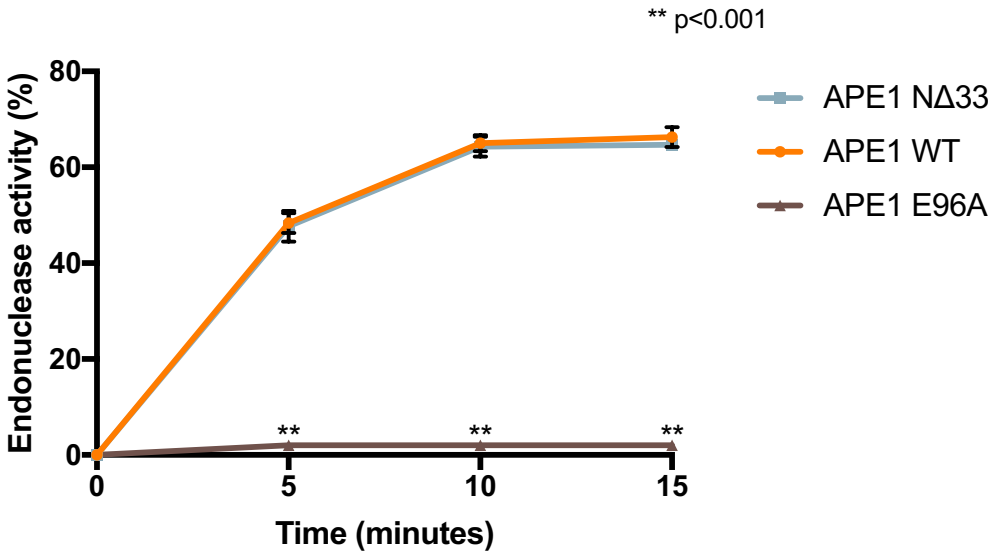
a)



b)



c)



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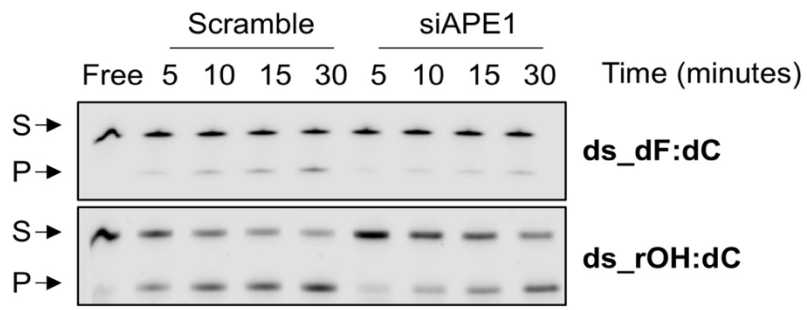
In order to confirm that APE1 is the major enzyme capable of cleaving ribo-abasic containing sites in cells, we used nuclear cell extracts in which APE1 was knocked-down through specific siRNAs (Figure 22), as explained in Material and Methods. As Figure 28 shows, the endonuclease activity of APE1-kd (siAPE1) cell extracts, was reduced on both ds_dF:dC and ds_rOH:dC as compared to its respective control SCR-treated extracts. Moreover, what emerges from the Western blot analysis (Figure 22), the expression of APE1 protein did not exert any effect on the expression of RNase H2 protein itself, demonstrating that the observed reduction of the processing activity of the abasic rNMP-containing substrate, observed with APE1-kd cell extracts, was likely due to the reduced expression of the APE1 protein.

These data demonstrate that human APE1 is the major enzyme capable of specifically cleaving at abasic rNMPs in DNA while being unable to process normal rNMPs, which are the preferential substrates of RNase H2.

Figure 28 ***APE1 knock down in human cells impairs the processing of a rAP site embedded in a duplex DNA substrate***

a) *Representative denaturing polyacrylamide gel of oligonucleotides (0.25 μM) incision by nuclear HeLa cell extracts (NCE). In order to discriminate the activity of APE1, 10 ng of NCE, in which APE1 expression was previously knocked down through specific siRNA (indicated as siAPE1), were tested in comparison to control cells (Scramble) at different time points, expressed in minutes and shown on the top of the figure. dF- containing oligonucleotide was used as positive control. Enzymatic reaction was performed at 37°C in APE1-buffer. S indicates the substrate position, while P indicates the product position.* ***b)*** *Graph illustrating the time-course kinetics activity of NCE on ds_dF:dC in control and APE1-knocked down conditions. Enzymatic reaction was performed at 37°C in APE1-buffer with 10 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas. Statistical significant differences, calculated through t-Test analysis, are indicated as *p<0.05 and **p<0.001.* ***c)*** *Graph illustrating the time-course kinetics activity of NCE on ds_rOH:dC oligonucleotide in control and APE1-knocked down conditions. Enzymatic reaction was performed at 37°C in APE1-buffer with 10 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas. Statistical significant differences, calculated through t-Test analysis, are indicated as *p<0.05 and **p<0.001.*

a)



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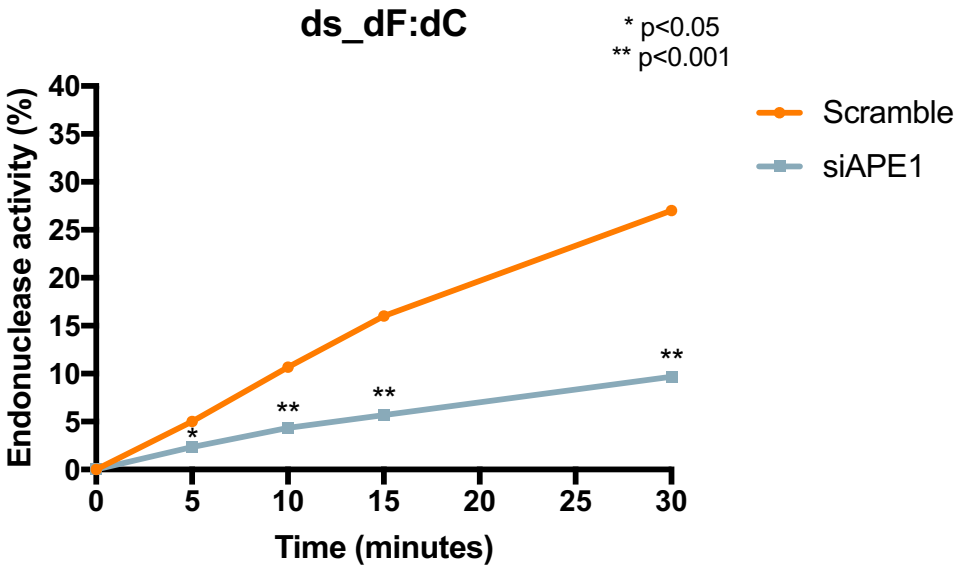

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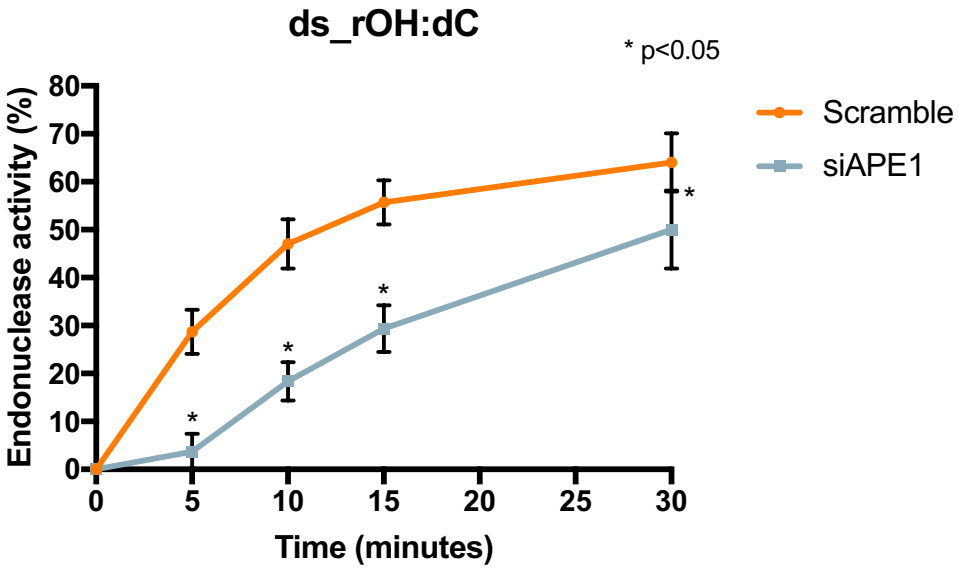

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Again, as above, the specificity of the enzymatic activity of APE1 was tested using the tetrahydrofuran ribonucleotide mimicking an abasic residue embedded in a longer DNA sequence. Moreover, a pre-treatment of Compound #3 (i.e. N-(3-(benzo[d]thiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl)acetamide)), a specific APE1 endonuclease inhibitor (Poletto, Malfatti, et al. 2016; Rai et al. 2013), was used. All these experiments confirmed that the main enzymatic activity on abasic ribonucleotides embedded in DNA is due to APE1 (*data not shown*) (Malfatti et al. 2017).

After demonstration that the ability of APE1 to cleave an abasic ribonucleotide embedded in DNA, and discarded the possibility of RNase H2 can have a role in the processing of this type of lesion, we hypothesized that ribonucleotides from DNA or from the nucleotide pool can be hydrolyzed spontaneously thus generating an abasic site. Another possibility can be the presence of an oxidized rNMP, that once processed by specific enzymes, is converted to abasic site, as in the case of the classic d8oxoG.

For this reason, we decided to focus our attention on r8oxoG removal. Experiments carried on this new type of lesion are presented in next paragraphs.

7.3 Human RNase H2 does not process a r8oxoG embedded in a duplex DNA

Abasic ribo- site can be generated by spontaneous hydrolysis of ribonucleotides embedded in DNA. Moreover, the processing of oxidized ribonucleotides embedded in DNA might generate abasic ribo- sites.

Thus, in the second part of the Project, we focused our attention on the processing of 8oxoG-rNMPs embedded in DNA.

The r8oxoG containing oligonucleotide was in-house synthesized and, as observed from MALDI-MS analysis and HPLC purification, the undesirable presence of secondary products of the chemical synthesis of this substrate can be excluded (Figure 17).

First of all, we investigated whether recombinant human RNase H2 protein was able to recognize and cleave at r8oxoG site using an oligonucleotide containing this type of lesion called ds_r8oxoG:dC (Figure 16 and Figure 29). In these experiments, we compared the specific enzymatic activity of RNase H2 with that exerted on the canonical ds_rG:dC substrate, as positive control, and using the ds_d8oxoG:dC oligonucleotide as negative control (Figure 29).

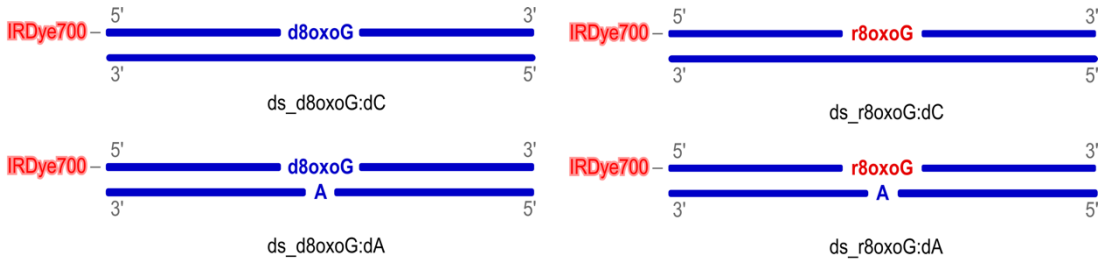


Figure 29 ***Scheme of substrates used to test the processing of a r8oxoG site embedded in a duplex DNA substrate***

Scheme of double-stranded (ds) DNA substrates containing a d8oxoGMP and a r8oxoGMP sites in the 13th position annealed to ss_dC containing- or ss_dA containing-complementary oligonucleotides. The 'IRDye700' in red indicate IRDye 700 phosphoramidite dye tagged at the 5' end. DNA nucleotides are in blue, RNA in red. The 5' and 3' ends of each DNA strand are indicated.

As Figure 30 shows, the ds_rG:dC oligonucleotide was efficiently processed by RNase H2 whereas the same activity was not observed for ds_r8oxoG:dC. As expected, the d8oxoG-containing oligonucleotide was not

cleaved by RNase H2. These data confirm that RNase H2 was not able to process modified rNMPs embedded in DNA. Further studies are ongoing in order to understand if RNase H2 might bind the oxidized rNMP.

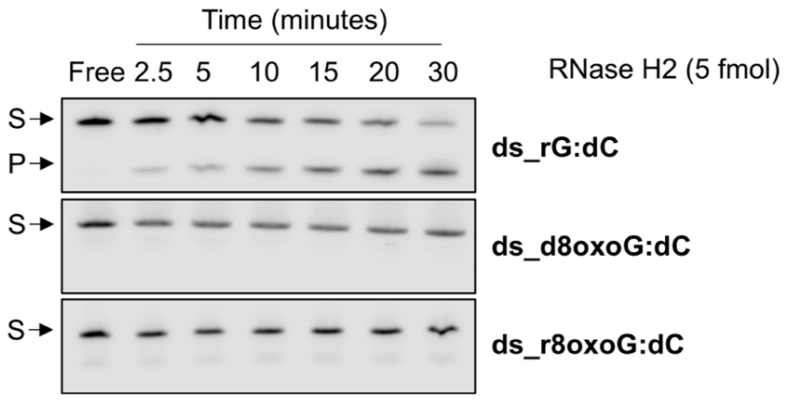


Figure 30 ***Recombinant human RNase H2 protein is not able to process a r8oxoG site embedded in a duplex DNA substrate***

Representative denaturing polyacrylamide gel of oligonucleotides (25 nM) incision by human recombinant RNase H2 (5 fmol). Reaction was performed in RNase H2-buffer (20 mM Tris-HCl, 25 mM KCl, 0.1% BSA, 0.01% Tween20, 4 mM $MgCl_2$, pH 7.4) at 37°C. ds_rG:dC and ds_d8oxoG:dC oligonucleotides were used as positive and negative controls, respectively. Time points are shown on the top of the figure. S indicates the substrate position, while P indicates the product position.

Similarly, we confirmed these data using RNase H2-kd nuclear extracts from HeLa cells (Figure 22). As expected, control nuclear extracts (Scramble) displayed a time-dependent endoribonuclease activity on ds_rG:dC, whereas the down regulation of RNase H2 protein expression (siRNase H2) was associated with a marked reduction of the endoribonuclease activity on the same substrate (Figure 31). On the contrary, once we tested the ability of the nuclear extracts on ds_r8oxoG:dC oligonucleotide, we found only a weak endoribonuclease activity on it , which was not affected by RNase H2 silencing (Figure 31).

These data support the conclusion that the r8oxoG site in DNA is not recognized by human RNase H2.

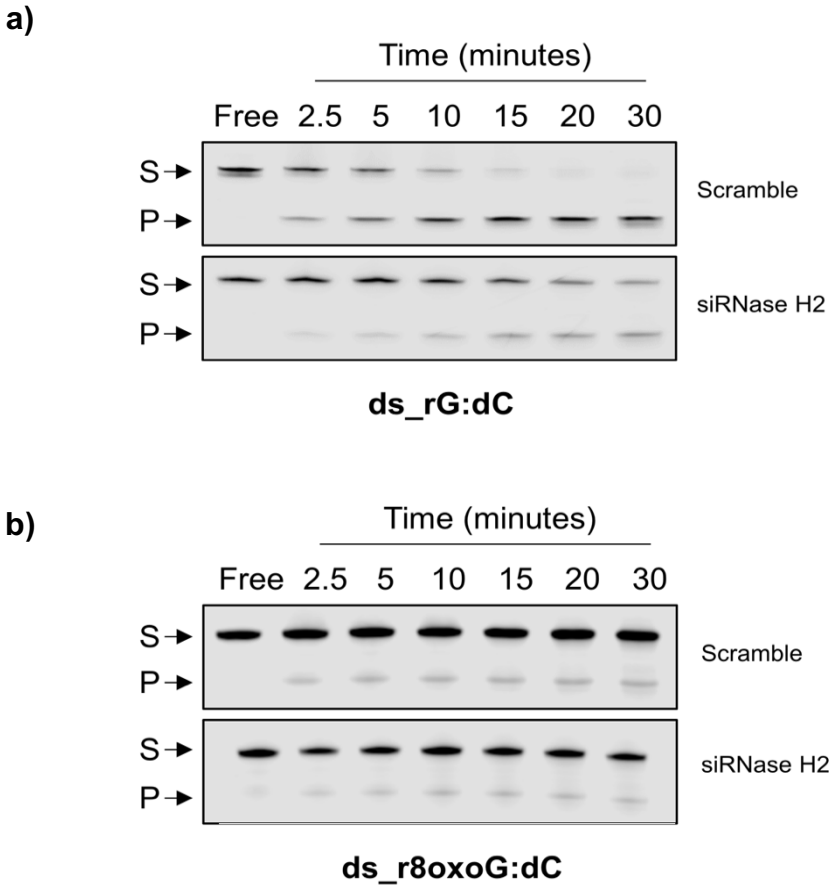


Figure 31 ***RNase H2 from cell extracts is unable to process a r8oxoG site embedded in a duplex DNA substrate***

a) *Representative denaturing polyacrylamide gel of ds_rG:dC and* ***b)*** *ds_r8oxoG:dC oligonucleotides (0.25 μM) incision by nuclear HeLa cell extracts (NCE). In order to discriminate the activity of RNase H2, 500 ng of NCE in which RNase H2 expression was previously knocked down through specific siRNA (indicated as siRNase H2) were tested in comparison to control cells (Scramble) at different time points (minutes), shown on top of the figure. S indicates the substrate position while P indicates the product position.*

7.4 OGG1 has neither lyase nor glycosylase activities on oxidized rG substrate

We then investigated whether enzymes of the BER pathway may be involved in the processing of the r8oxoG substrate. To this purpose, we tested recombinant purified human OGG1 and APE1 proteins (Figure 32 and Figure 24).

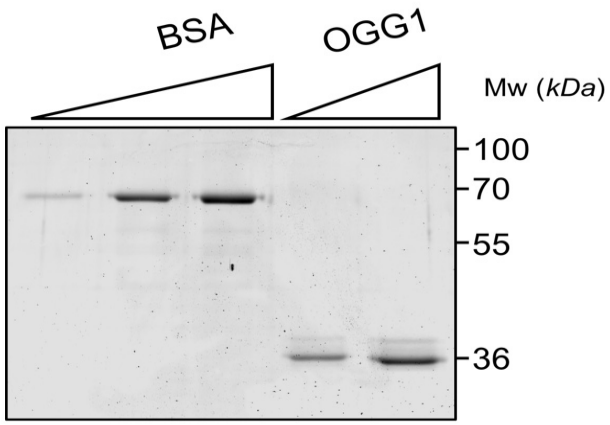


Figure 32 ***Gel-quantification of recombinant human OGG1 protein***

Increasing doses (250-500 ng) of human recombinant OGG1 protein was separated onto 10% SDS–PAGE gel followed by Coomassie staining. Bands corresponding to each protein were quantified and normalized on a standardization curve (250-500-750 ng) of Bovine Serum Albumin (BSA) protein. The molecular weight (Mw) expressed in kilodaltons (kDa) is shown on the right of each panel.

As explained in the Introduction section, OGG1 protein belongs to the bi-functional glycosylases family having both lyase and glycosylase activities on oxidized dG (Boiteux and Radicella, 2000). Thus, we examined the processing activity of OGG1 on the r8oxoG substrate in comparison to the d8oxoG-containing oligonucleotide, as a positive control (Figure 29). First, we tested the ability of OGG1 to recognize an r8oxoG site through electrophoretic mobility shift assay (EMSA). As shown in Figure 33, increasing amount of recombinant OGG1 formed a stable retarded complex with the r8oxoG oligonucleotide in a dose-dependent manner. In addition, as confirmed, OGG1 binding was specific for the modified r8oxoG- containing oligonucleotide (*lanes 2 and 3*). As expected, the shift was observed when recombinant OGG1 was incubated with the positive control ds_d8oxoG:dC (*lanes 11 and 12*) but not with the negative controls ds_rG:dC (*lanes 5 and 6*). A weak signal is detected

when the ds_dG:dC oligonucleotide was used (*lanes 8 and 9*) and is attributable to a low affinity binding of the enzyme to the unmodified DNA (Figure 33).

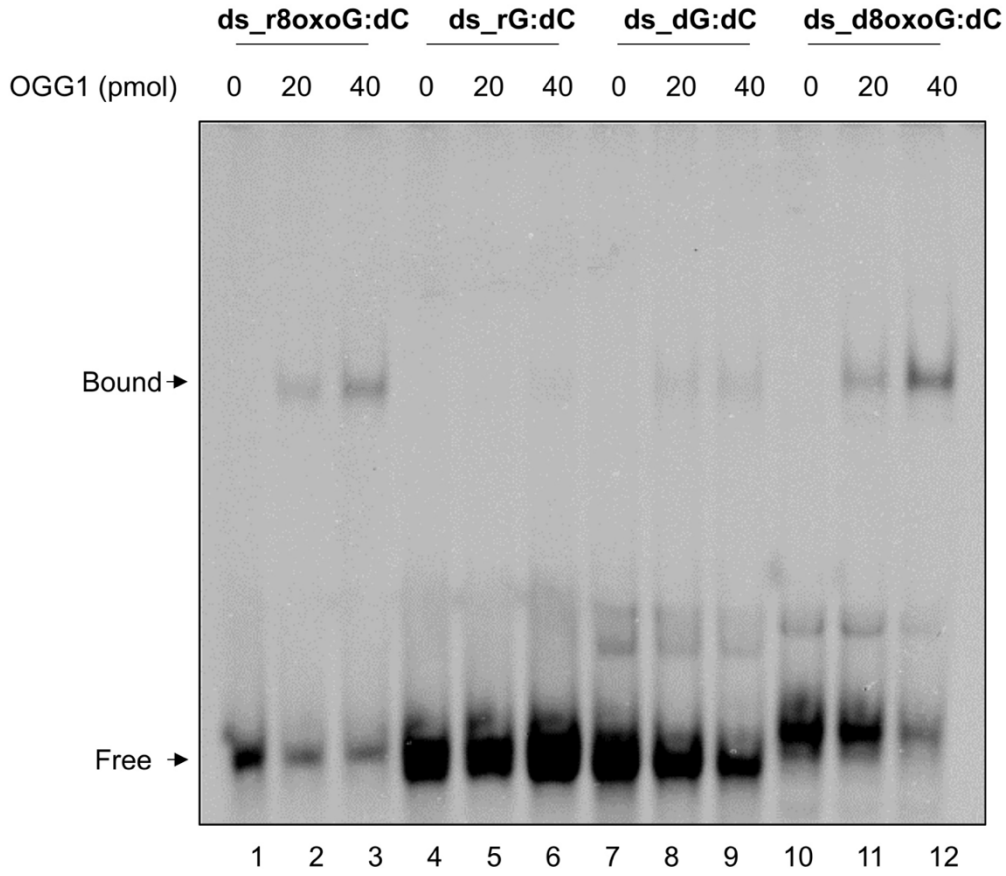


Figure 33 ***Human OGG1 binds r8oxoG- containing oligonucleotide***

Representative native EMSA polyacrylamide gel of OGG1 binding on ds_r8oxoG:dC oligonucleotide (25 nM) is shown. ds_dG:dC and ds_rG:dC are used as negative controls whereas ds_d8oxoG:dC is used as positive control. *The 'Bound' arrow indicates the retarded complex between OGG1 and the probe whereas the 'Free' arrow the unbound substrate. Amounts of OGG1 protein, expressed in pico moles, are shown on the top of the figure. Reactions were performed as explained in "Material and Methods" section.*

We next tested the lyase activity of OGG1 on different substrates. Figure 34 shows that OGG1 was only able to process the canonical substrate ds_d8oxoG:dC in a dose response manner. In contrast, no lyase activity was apparent for any of the other substrates used, including the ds_r8oxoG:dC and the ds_r8oxoG:dA.

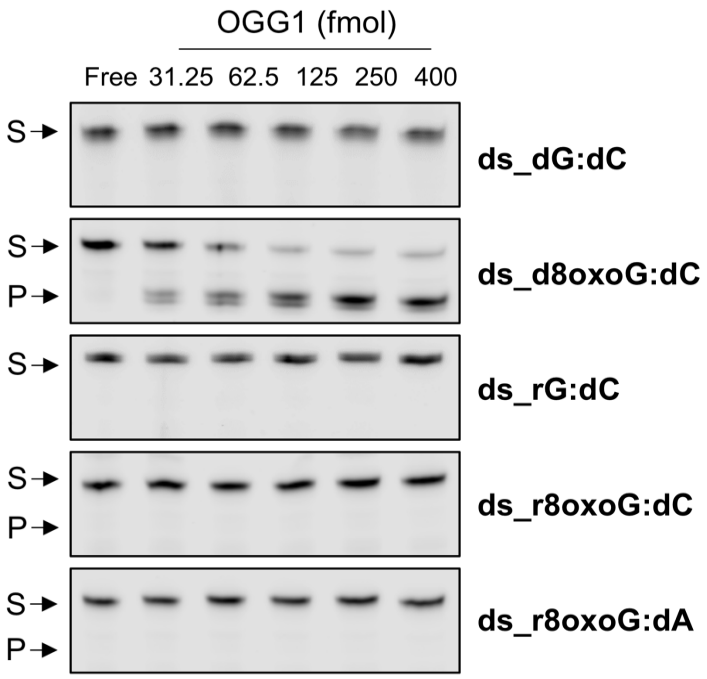


Figure 34 ***Human OGG1 has not any lyase activity on r8oxoG-containing oligonucleotide***

Representative denaturing polyacrylamide gel of lyase activity of human OGG1 on different duplex DNA oligonucleotides (25 nM). Doses of OGG1 protein expressed in femto moles are shown on the top of the figure. Reactions were performed in OGG1-buffer (20 mM Tris-HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4) at 37°C for 30 minutes. S indicates the substrate position, while P indicates the product position.

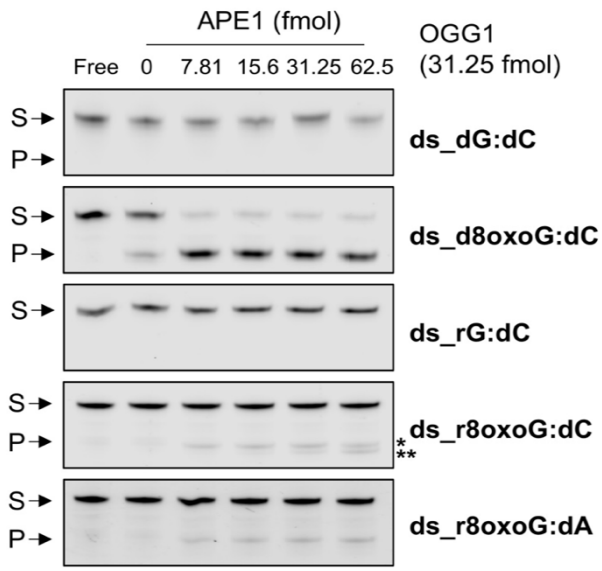
OGG1 is the major glycosylase enzyme in the BER pathway, able to convert oxidized bases in abasic sites, subsequently cleaved by the endonuclease APE1 (Krokan and Bjoras 2013; Boiteux and Radicella 1999; David, O'Shea, and Kundu 2007). In order to measure its glycosylase activity on the same substrates, we co-incubated a fixed amount of recombinant OGG1 with increasing amounts of recombinant purified APE1 (Figure 35). While OGG1 displayed a robust glycosylase activity on the canonical ds_d8oxoG:dC substrate, a weak activity on ds_r8oxoG:dC oligonucleotide (indicated by a single asterisk) was observed (Figure 35). Moreover, the presence of an additional higher mobility band, increasing as a function of APE1 concentration (indicated with a double asterisk) was observed only in the case of the ds_r8oxoG:dC substrate.

We conclude that OGG1 has neither lyase nor glycosylase activity on the r8oxoG substrate, and that APE1 can weakly process this substrate alone. About APE1 activity, a detailed description will be explained in the next paragraph.

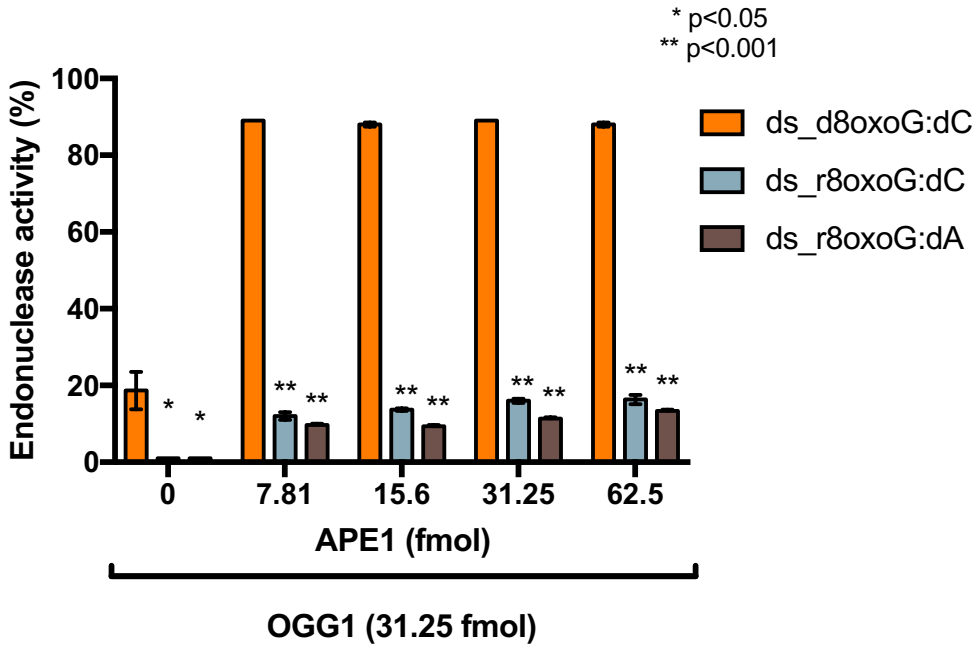
Figure 35 ***Human OGG1 does not possess a glycosylase activity on r8oxoG-containing oligonucleotide***

a) *Representative denaturing polyacrylamide gel of incision by different doses of APE1 co-incubated with a fixed amount of OGG1 (3.125 nM) on different duplex DNA oligonucleotides (25 nM) in order to investigate glycosylase activity of OGG1. Different doses of APE1 protein, expressed in femto moles, are shown on top of the figure. Reactions were performed in a buffer containing 20 mM Tris-HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4 at 37°C for 30 minutes. S indicates the substrate position while P indicates the product position. Moreover, at the right of the panel, a longer product of about 12 nucleotides is indicated by an asterisk whereas a smaller one of 11 nucleotides is indicated by a double asterisk.* ***b)*** *Histograms represent the dose response of OGG1 glycosylase activity on ds_d8oxoG:dC and paired and mismatched ds_r8oxoG oligonucleotides. ds_d8oxoG oligonucleotide was used as a positive control whereas ds_dG:dC and ds_rG:dC oligonucleotides were used as negative controls. The activity is reported as percentage of substrate converted to product. Data are expressed as mean ± SD of three independent technical replicas. Statistical significant differences, calculated through t-Test analysis, are indicated as *p<0.05 and **p<0.001.*

a)



b)



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7.5 APE1 has a weak endo-/exo-nuclease activities on the r8oxoG-containing substrate depending on Mg^{2+} concentration and on the presence of its N-terminal domain

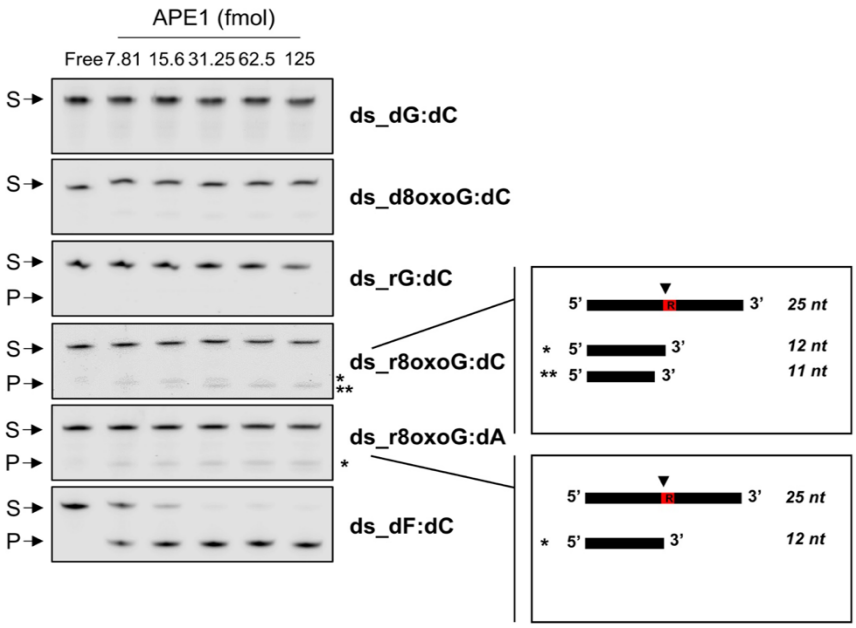
Based on the above presented data, we then checked whether APE1 '*per se*' had any endoribonuclease activity on ds_r8oxoG:dC substrate (Figure 36). Compared to the ds_dF:dC substrate, APE1 displayed a modest, though significant, processing activity on both ds_r8oxoG:dC and ds_r8oxoG:dA oligonucleotides, while no activity was observed in the case of the dG- and the d8oxoG-containing substrates (Figure 36), as expected. As observed above, the appearance of an additional faster migrating cleavage product (indicated by a double asterisk corresponding to a 11-nt product) was visible in the case of the ds_r8oxoG:dC substrate, which might be associated with a recently identified 3'-exonuclease activity by the protein (Chohan et al. 2015).

In contrast, using the oligonucleotide containing the mismatched ds_r8oxoG:dA, most of the fragments produced after incision by the AP endonucleolytic activity (indicated with a single asterisk) were not further degraded by the exonucleolytic activity.

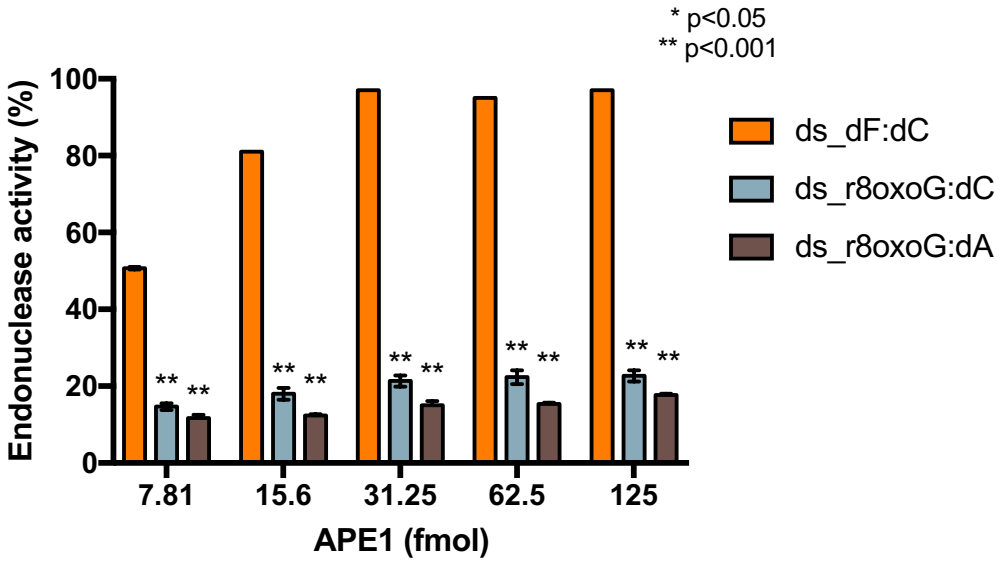
Figure 36 ***Human APE1 shows weak endo- and 3'-exonuclease activities on the r8oxoG substrate***

a) *Representative denaturing polyacrylamide gel of APE1 incision on different duplex DNA oligonucleotides (25 nM), in which ds_dF:dC oligonucleotide was used as a positive control, whereas ds_dG:dC and ds_rG:dC oligonucleotides were used as negative controls. The doses of APE1 protein used, expressed in femto moles, are shown on the top of the figure. On the right side, a schematic representation of the cleavage products, showing the position of the ribonucleotide (red box with R) embedded in the DNA oligonucleotide and the APE1 cleavage on it, producing a longer product of about 12 nucleotides (*) and a smaller one of 11 nucleotides (**). Reactions were performed in APE1-buffer containing 20 mM Tris-HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4 for 30 minutes at 37°C. S indicates the substrate position while P indicates the product position.* ***b)*** *Relative graph indicates a dose-response APE1 activity on paired and mismatched ds_r8oxoG:dC oligonucleotide in comparison to ds_dF:dC positive control. Data are expressed as mean ± SD of three independent technical replicas. Statistical significant differences, calculated through t-Test analysis, are indicated as **p<0.001.*

a)



b)



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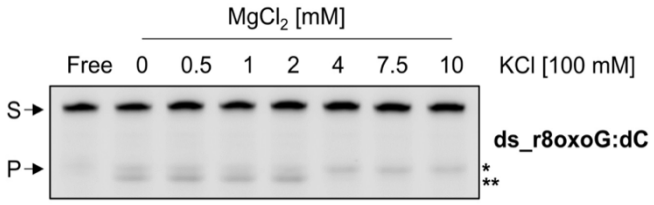
Data *not shown,* in which the missing cleavage on single stranded oligonucleotide (ss_r8oxoG) by APE1 was compared to the weak cleaved product of the annealed oligonucleotide (ds_r8oxoG:dC), demonstrated the requirement for secondary structured oligonucleotide sequences for efficient enzymatic activities by APE1. These data thus exclude that the observed cleavage product was due to the processing of a residual non-annealed oligonucleotide possibly present after the annealing reaction (Malfatti et al. 2017).

Together, these data suggest that the APE1 enzymatic activity on the r8oxoG substrate requires a dsDNA molecule and the exonuclease activity is dependent on the paired nucleotide, possibly as a consequence of a different stereo-chemical geometry between the 8oxoG:A and the 8oxoG:C.

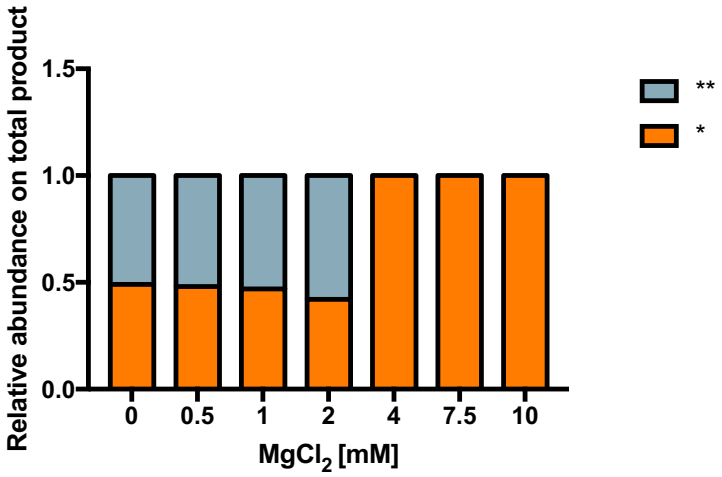
It has been previously demonstrated that the exonuclease activity of APE1 strictly depends on salt concentrations (Chou and Cheng 2003). We therefore tested whether the 3'-exonuclease activity observed on the ds_r8oxodG:dC shared some common features (in terms of dependence on the ionic strength conditions) with the 3'-exonuclease activity on mispaired DNA, as previously described (Chou and Cheng 2003). Firstly, we determined the optimal $MgCl_2$ (Figure 37**a-b**) and KCl (Figure 37**c-d**) concentrations required for the 3'-exonuclease activity. The 3'-exonuclease activity was present up to a concentration of 2 mM $MgCl_2$. An inhibitory effect was apparent at $MgCl_2$ concentrations above 4 mM. At the same time, the 3'-exonuclease activity was poorly affected at KCl concentration equal to 100 mM.

These results are in line with previous data on 3'-mispaired DNA (Chou and Cheng 2003) and suggest that the observed 3'-exonuclease activity strongly depends on the electrostatic interaction of APE1 with the substrate during the cleavage reaction and with the role of Mg^{2+} ions.

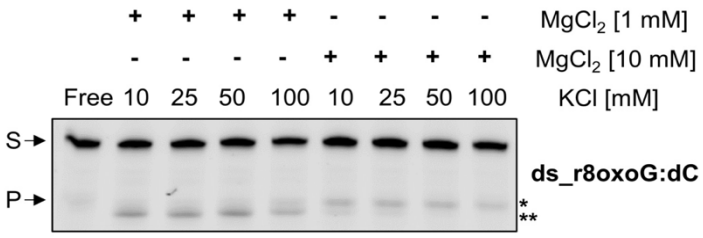
a)



b)



c)



d)

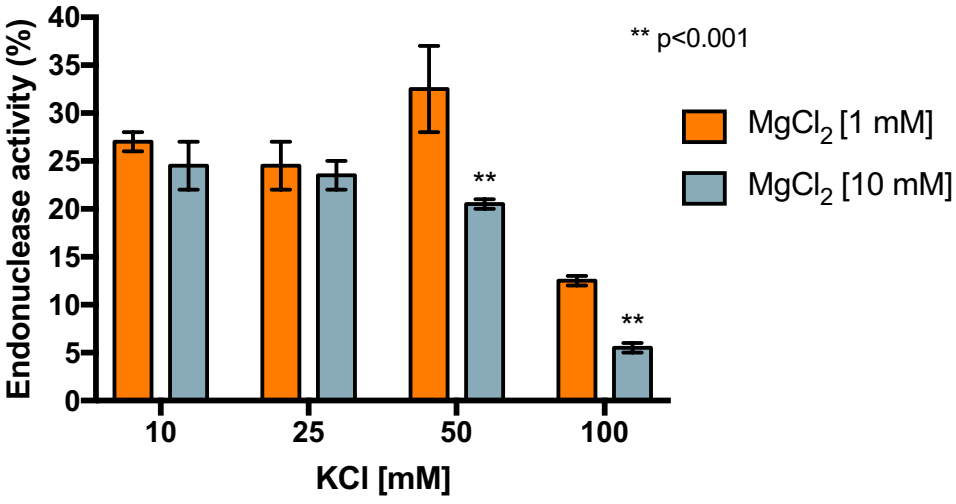


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Figure 37 ***Human APE1 activities on the r8oxoG substrate depend on mono- and di-valent cations***

a*) Representative denaturing polyacrylamide gel of APE1 (5 nM) incision on ds_r8oxoG:dC oligonucleotide under different $MgCl_2$ concentrations that is expressed in mM (top) performing a reaction long 30 minutes in APE1 buffer. S indicates the substrate position while P indicates the product position. Moreover, at the right of the panel, a longer product of about 12 nucleotides is indicated by an asterisk whereas a smaller one of 11 nucleotides is indicated by a double asterisk.* ***b)*** *Relative graph shows the ratio between two products obtained as a function of $MgCl_2$ concentration.* ***c****) Representative denaturing polyacrylamide gel of APE1 (5 nM) incision on ds_r8oxoG:dC oligonucleotide under different KCl concentrations in combination with two different $MgCl_2$ concentrations, 1 mM and 10 mM (top) performing a reaction long 30 minutes in a buffer containing 20 mM Tris–HCl, 0.1% BSA, 0.01% Tween20, pH 7.4. S indicates the substrate position, while P indicates the product position. Moreover, at the right of the panel, a longer product of about 12 nucleotides is indicated by an asterisk whereas a smaller one of 11 nucleotides is indicated by a double asterisk.* ***d)*** *Relative graph shows product levels in association with different salts concentrations. Statistical significant differences, calculated through t-Test analysis, are indicated as **p<0.001.*

After choosing the optimal salts conditions, in which both endo- and exo- activities of APE1 (100 mM KCl and 1 mM $MgCl_2$) are present, we evaluated whether the enzymatic activity of APE1 on ds_r8oxoG:dC was dependent on the same catalytic site responsible for the endonuclease activity observed on abasic dsDNA and abasic rNMP within dsDNA (*data not shown*) (Malfatti et al. 2017). To this aim, the enzymatic activity of the E96A mutant was compared to that of the WT protein. These data demonstrate that the APE1 E96A mutant has a reduced endoribonuclease activity showing no 3'-exonuclease activity over the ds_r8oxoG:dC substrate. Due to the effect of salt concentration on this latter activity, we also tested the enzymatic activity of the APE1 NΔ33 deletion mutant. Interestingly, while this protein retained the endoribonuclease activity of the WT protein, its 3'-exonuclease activity was abolished. Moreover, treatment with APE1 inhibitor Compound #3 confirmed that the catalytic site responsible for the endonuclease activity is also responsible for the endoribonuclease activity over the r8oxoG substrate (*data not shown*) (Malfatti et al. 2017).

These results show that APE1 has a weak, though significant, endoribonuclease activity on the r8oxoG substrate with an additional specific 3'-exonuclease activity dependent on:

i) the kind of base pairing: i.e. ds_r8oxoG:dC or ds_r8oxoG:dA;

ii) salt concentrations (i.e. Mg^{2+});

iii) the presence of the 33 N-terminal domain.

Overall, our data suggest that BER enzymes, but not RER, are involved in the processing of non-canonical rNMPs, such as abasic or oxidized, incorporated in DNA. Because the repair pathway catalyzed by APE1 towards the oxidized rG is fundamentally different from that of BER, since no bases are excised, we can conclude that the observed effect could be ascribed to an alternative damage-specific endonuclease initiated repair pathway, previously referred to as either alternative excision repair (AER) or nucleotide incision repair (NIR) (reviewed by (Yasui 2013) and by (Prorok et al. 2013)).

7.6 APE1 and RNase H2 do not biochemically and functionally interact in human cells

In the last part of this Thesis, we performed preliminary experiments to support the functional independence between the BER and the RER pathways.

First, we tested whether APE1 and RNase H2 proteins functionally interact. We used HeLa cells transfected with siRNAs specific for APE1 and RNase H2 mRNAs to knock down the corresponding endogenous proteins. Whole cell extracts were prepared as explained in Materials and Methods section, and Western blotting analysis was used to check the effective protein down regulation (Figure 38).

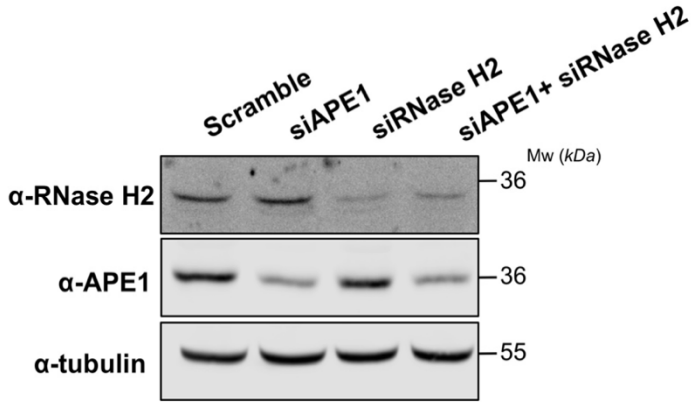


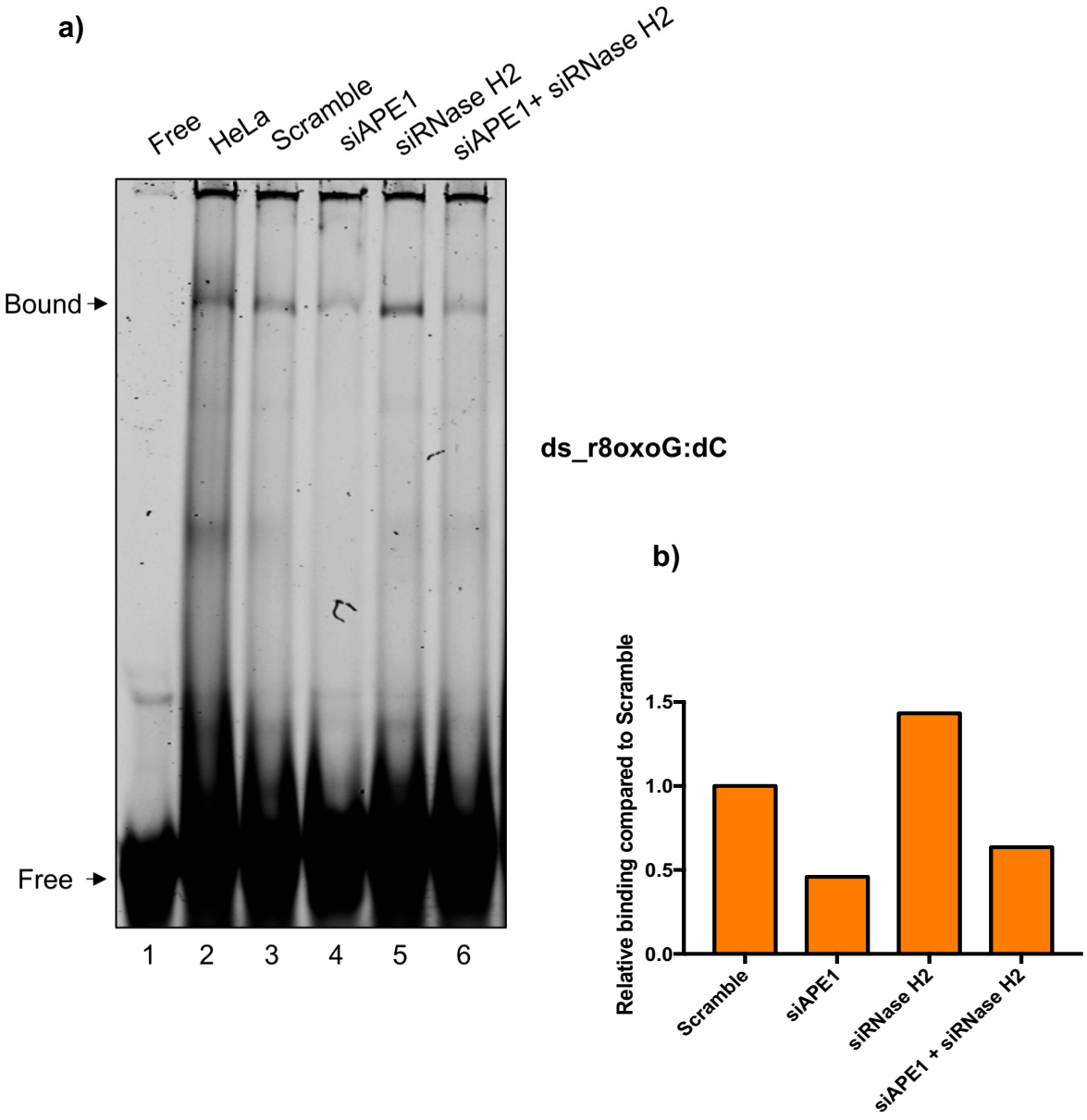
Figure 38 ***Western blot analysis of whole cell extracts silenced for APE1 and RNase H2***

HeLa cells were transfected with siRNAs directed versus RNase H2 and APE1 proteins and compared to the Scramble control. Whole cell extracts were separated onto 12% SDS–PAGE, and Western blot analysis was performed by using an anti-APE1 antibody and an anti-RNase H2 antibody. Tubulin was used as loading control. The molecular weight (MW) expressed in kilodaltons (kDa) is shown on the right of each panel.

Then, we checked the ability of APE1 and RNase H2 from cell extracts to recognize the ds_r8oxoG:dC substrate. We performed EMSA analysis with cell extracts from control (Scramble) and APE1-kd (siAPE1) or RNase H2-kd (siRNase H2) cells. As demonstrated in Figure 39**a**, incubation of cell extract from control cells displayed a shift containing APE1-complex. The intensity of the retarded complex was decreased upon APE1-kd (siAPE1) (Figure 39**a**,

lane 4) and upon the double APE1/RNase H2-kd (siAPE1 + siRNase H2) (Figure 39**a**, *lane 6*) but not upon RNase H2 silencing alone (siRNase H2) (Figure 39**a**, *lane 5*). This suggest that APE1 is involved in a protein complex able to recognize r8oxoG damage in which RNase H2 is not present.

These data were also confirmed by *supershift* EMSA experiments with anti-APE1 specific antibody in which the "Bound" signal was supershifted in the presence of antibody directed *versus* APE1, confirming the presence of APE1 in the binding complex of r8oxoG damage (Figure 39**c**).



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c)

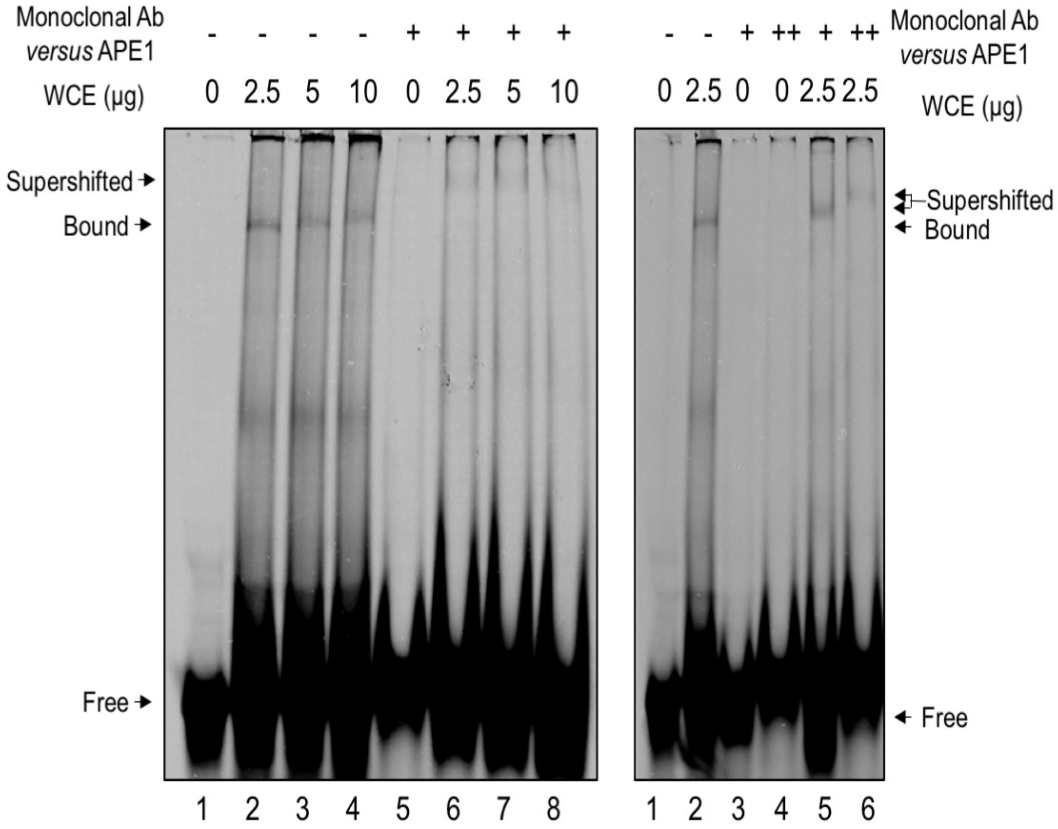

Figure 39 ***APE1 is part of the protein complex recognizing r8oxoG damage from cell extracts***

a) *Representative native EMSA polyacrylamide gel on ds_r8oxoG:dC oligonucleotide incubated with 2.5 µg of whole cell extracts from untreated HeLa cells (lane 2) and HeLa cells transfected with control siRNA (lane 3), silenced for APE1 (lane 4), RNase H2 (lane 5) and APE1/RNase H2 (lane 6) for 30 minutes at 37°C. Lane 1 corresponds to r8oxoG-containing oligonucleotide alone without any cell extract. On the right side of the gel, an arrow denotes the retarded complex between cell extract and the probe (left).* ***b)*** *Histogram shows the difference of binding between each condition.* ***c)*** *Representative native polyacrylamide gel of ds_r8oxoG:dC oligonucleotide incubated at 37°C for 30 minutes with the indicated amounts of whole cell extracts (WCE) without or with a pre-incubation with the specific monoclonal antibody recognizing APE1 protein, at 4°C for 1 hour, before gel separation. Bound complex to the oligonucleotide is indicated by an arrow named 'Bound', whereas the supershifted complex, obtained after incubation with the specific antibody, is indicated by an arrow named 'Supershifted'. Representative native polyacrylamide gel of ds_r8oxoG:dC oligonucleotide incubated at 37°C for 30 minutes with the indicated amounts of whole cell extracts (WCE) without or with a pre-incubation with increasing amounts of the specific monoclonal antibody recognizing APE1 protein, at 4°C for 1 hour, before gel separation. Bound complex to the oligonucleotide is indicated by an arrow named 'Bound', whereas the supershifted complex, obtained after incubation with the specific antibody, is indicated by an arrow named 'Supershifted'.*

8. Discussion

The incorporation of rNMPs within DNA is a frequent phenomenon occurring into the genome of both prokaryotes and eukaryotes (Williams and Kunkel 2014; Koh, Balachander, et al. 2015; Hovatter and Martinson 1987; Potenski and Klein 2014). The presence of rNMPs into the genome causes genomic instability, but the cell responds with a specific pathway, called RER pathway, in which RNase H2 works as endonuclease enzyme, able to cleave at the 5' side of the rNMP (Sparks et al. 2012; Heider et al. 2017). That RNase H2 functionality is important within the cell is testified by its requirement for the embryonic development in mouse (Hiller et al. 2012) and by the fact that, when mutated, is causally linked to the onset of AGS, a rare autoimmune inflammatory disease (Rice et al. 2013; Pizzi et al. 2014).

Until now, Topoisomerase I is the only enzyme able to cleave rNMPs embedded in DNA, when the RER pathway does not work. Contrarily, studies conducted on the MMR and the NER pathways have shown the inefficiency of these pathways in the processing of this particular lesion (Williams et al. 2013; Lindsey-Boltz et al. 2015). It is still unclear if other known DNA repair pathways work as back-up mechanisms of the RER pathway.

Remarkably, considering several published data, it can be hypothesized that, among many millions rNMPs that are introduced in the mammalian genome per cell cycle (Williams, Lujan, and Kunkel 2016), damaged rNMPs (such as abasic and oxidized) can also be incorporated into the DNA. In fact, RNA molecules, as well as rNMPs present in the nucleotide pool, are also susceptible to oxidative insults (Randerath et al. 1992; Moreira et al. 2008). Moreover, a significant generation of abasic sites formation has been demonstrated upon RNA oxidation and alkylation (Loeb and Preston 1986).

For this reason, whereas the role of RNase H2-initiated RER mechanism of DNA repair in recognizing and cleaving rNMPs embedded in DNA is well established (Williams, Lujan, and Kunkel 2016; Cerritelli and

Crouch 2009), little is known if the RER pathway, or other DNA repair pathways are involved in the removal of damaged rNMPs.

Describing the repair of this non-canonical lesions could be considered a great step forward in the DNA repair field.

In this context, a role of the Base Excision Repair (BER) pathway has not been addressed, yet. BER is the main pathway coping with the repair of non-distorting single-base lesions, such as abasic sites and oxidized bases (Dianov et al. 2001). For its abilities, BER may represent an interesting candidate to work as a back-up mechanism of RER on canonical rNMPs in DNA as well as preferable candidate on repairing abasic or oxidized rNMPs within DNA. Moreover, this hypothesis is encouraged by new discovered functions of the BER pathway in the RNA quality control surveillance and RNA-decay. Specifically, a role of APE1, the main AP endonuclease working in the BER pathway, emerges for its ability to cleave abasic damaged RNA and for its involvement in the processing of miRNAs (Tell, Wilson, and Lee 2010; Jobert and Nilsen 2014; Antoniali et al. 2017).

Although great steps forward have been done along this direction, there is still no evidence that the BER pathway can be associated to the removal of unmodified and/or modified rNMPs embedded in DNA. Characterizing this new unexpected ability of BER enzymes could improve our knowledge around the genotoxicity mechanism of oxidative stress and the impact of BER defects in human disease, cancer mechanisms, and for the development of new anticancer strategies.

In light of this, this Project points on investigating if the RER pathway has a role on repairing abasic and oxidized rNMPs within DNA and on improving our knowledge about the putative role of BER on unmodified and modified rNMPs embedded in DNA. In this context, by different *in vitro* assays and using different DNA oligonucleotides containing different damaged bases, we obtained interesting results that advance important information in this field.

First, we started from abasic rNMPs (rAP) site embedded in a DNA oligonucleotide. We demonstrate that the rAP site is targeted by APE1 rather than RNase H2 in eukaryotic systems. Specifically, we have found that

eukaryotic RNase H2 enzymes from yeast and mouse (*data not shown*) and human, are unable to process rAP sites in DNA, whereas recombinant human APE1 is able to efficiently cleave this type of damage. APE1 processes the rAP site as efficiently as the canonical deoxy-abasic site as measured by kinetic data. Data using catalytically inactive APE1 mutants (E96A) clearly demonstrate that the endonuclease active site of APE1 is required to perform the endoribonuclease activity on a ribose abasic site in dsDNA. Differently, the cleavage activity of the 33N-terminal truncated mutant, which does not impact the catalytic function of the enzyme but is involved in the release of the product upon cleavage (Fantini et al. 2010), is comparable to that of wild-type APE1. This last result demonstrates that the unstructured N-domain is dispensable for the enzymatic reaction on the abasic ribonucleotide site. Moreover, using RNase H2 or APE1 depleted HeLa nuclear extracts, we showed that the processing activity of the rAP site in DNA depends only on the presence of APE1 and not on RNase H2.

These results highlight a new role of APE1 in processing rAP sites embedded in DNA, demonstrating that the catalytic site of APE1 and the mechanism of product release is similar to that of the canonical deoxy-substrate.

In the second part of this Project, we moved on oxidized rG embedded in DNA. A potentially significant, yet poorly characterized, source of rNMPs incorporated in DNA may arise from oxidative stress. rNMPs were shown to form during oxidative DNA damage both *in vitro* and *in vivo* (Randerath et al. 1992). This means that oxidation can occur not only on the G base but also on the deoxyribose converting it in ribose. Moreover, it is be possible that abasic and oxidized dNMPs into DNA are converted into abasic or oxidized rNMPs embedded in DNA.

Furthermore, rAP sites embedded in DNA may be generated by spontaneous hydrolysis or by the action of an unknown glycosylase on oxidized rNMPs, such as r8oxoG.

Published data have estimated the rate of depurination in DNA under physiological conditions that is about 10,000 abasic sites per day in human cells (Lindahl and Nyberg 1972). As it happens for DNA, spontaneous

depurination also occurs in RNA, although ~ 1,000 times slower than DNA (Kochetkov and Budovskii 1972). Considering that rNMPs abundance in DNA is very high (around 600,000 rNMPs in budding yeast genomic DNA, and therefore a factor of 250 higher in mammalian genomic DNA (150,000,000) (Williams, Lujan, and Kunkel 2016)), it is not unrealistic to deduce that cells may contain a non-negligible number of rAP sites in DNA as well as oxidized rNMPs, caused by oxidative stressors to whom the cell is often exposed, and that can increase in cancer cells.

Interestingly, abasic RNA results significantly more stable than abasic DNA, suggesting that specific enzymatic mechanisms should exist *in vivo* to cope with this lesion (Kupfer and Leumann 2006).

In support of this, recent studies suggested that r8-oxoGTP is formed *in vivo* under oxidative stress conditions and may be incorporated during replication into DNA by *S. pombe* and *M. smegmatis* polymerases (Sastre-Moreno et al. 2014; Ordonez and Shuman 2014; Cilli et al. 2015), as well as by human DNA Polβ. However, concerning this last case, since the level and function of Polβ in cells are highly regulated by complex signaling mechanism (Parsons et al. 2011), the probability of r8oxoG incorporation into genomic DNA by Polβ is still a question under debate, which needs further experimental proof.

Because of the spontaneous formation of rAP sites is a rare event, it is a key question to find enzymatic activities able to generate rAPs starting by a previous processing of oxidized rNMPs embedded in DNA.

Although a possible RER and BER involvement in the removal of r8oxoG from DNA has been proposed in other reports (Cilli et al. 2015; Sassa et al. 2016), further work in our laboratory is ongoing along these lines to address these fundamental issues.

Considering all these observations, we investigated the role of the RER pathway in the recognition and cleavage of oxidized rNMPs (r8oxoG) within DNA. After demonstrating its inability, we focused on BER proteins.

First, we tested the RNase H2 activity on r8oxoG in a DNA substrate. Similarly to results obtained with the abasic rNMP in DNA, our data clearly demonstrate that the RER pathway is not involved in processing of oxidized rNMPs embedded in DNA. Eukaryotic RNase H2 is unable to process this

lesion *in vitro*. Interestingly, additional ongoing studies show that the prokaryotic counterpart of that protein seems to preserve the ability to process this modified rNMPs in DNA. Moreover, although the prokaryotic RNase HII is active on oxidized rG, the eukaryotic RNase H2 is completely inactive on this lesion, suggesting that the ability to process r8oxoG in DNA might have been lost during evolution.

Based on these observations, we explored a potential role of the BER proteins, including OGG1 and APE1. First, we showed that the human OGG1, the main glycosylase enzyme able to recognize and repair oxidized dG, has neither a glycosylase nor a lyase activity on oxidized rG site embedded in a DNA substrate.

Despite the ability of OGG1 to efficiently bind the oxidized substrate was confirmed, in agreement with recent findings (Sassa et al. 2016), it is possible to speculate that the enzyme is unable to hydrolyze the N-glycosidic bond of the r8oxoG maybe as a consequence of the steric hindrance with the 2'-OH of the ribose which renders the C1' unavailable for the nucleophilic attack by the catalytic site.

Interestingly, we discovered that APE1 has a weak endoribonuclease activity on r8oxoG site embedded in a DNA substrate, and shows a 3'-exonuclease activity, similarly to the 3'-exonuclease activity on DNA demonstrated previously (Wilson 2005; Beernink et al. 2001). In line with previous results, the 3'-exonuclease activity of APE1 is strictly dependent on Mg^{2+} concentration and on the presence of the first 33 aminoacids. The importance of the N-terminal domain is explained by different observations: *i)* it bears the majority of the positive charges of APE1; *ii)* is the target of the main post-translational modifications of the protein (i.e. acetylation, ubiquitination, proteolysis); *iii)* is involved in modulating the interaction with different protein partners, and finally *iv)* may modulate the catalytic rate, probably acting on the k_{off} of the catalytic reaction due to increased speed of product release (Vascotto et al. 2009; Fantini et al. 2010). These unexpected results, which suggest that APE1 3'-exonuclease activity strongly depends on the electrostatic interaction of APE1, involving its unstructured N-terminal domain, with the substrate (Wilson 2005), may be explained on the basis of the

previously characterized ability of APE1 to process some particular structured RNA species in a specific manner (Poletto et al. 2013). The weak endo- and 3'-exonuclease activities on r8oxoG- containing substrate and their dependence on Mg^{2+}-concentrations and on the presence of the first N-terminal domain residues of APE1 are fully in agreement with the previously described nucleotide incision repair (NIR) function by APE1 on several oxidized substrates, such as: 5,6-dihydro-2'-deoxyuridine, 5,6-dihydrothymidine, 5-hydroxy-2'-deoxyuridine, 5-hydroxycytosine (Mazouzi et al. 2013; Gros 2004b; Daviet et al. 2007b). Notably, the limited activity obtained in the experimental conditions we used (also after changing the pH conditions, *data not shown*) are in agreement with previous reports on the NIR function by APE1 on some particular substrates, such as the αdG:dG and the 5OH-dC:dG (Daviet et al. 2007b). In addition, the biochemical characterization through MALDI-MS and HPLC analyses we performed may be suggestive for the existence of an equilibrium between different conformational species of r8oxoG dsDNA, excluding any possible bias due to contaminant present in the oligonucleotide used for the assays.

Differently from our results, Sassa *et al*., found no enzymatic activity by APE1. This discrepancy with our results may be due to the different experimental conditions for the enzymatic assays, i.e. higher Mg^{2+} concentrations and the use of a small amount of EDTA, both aspects already demonstrated to strongly affect the APE1 enzymatic activity on the r8oxoG substrate. A comparative experiment, we performed (*data not shown*), was indeed supportive of this hypothesis, reinforcing the importance of the experimental conditions when studying the non-canonical functions of APE1 protein.

Therefore, these findings underscore the importance of identifying which enzyme(s) are responsible for the recognition and the efficient processing of the r8oxoG substrate, in order to further extend our studies of this hot scientific topic.

The model structures of DNA with a unmodified rNMP or a rAP site in the RNase H2 and APE1 active site, respectively were compared (Figure 40). The canonical rNMP base is hydrogen bonded to the complementary DNA

strand base (Rychlik et al. 2010). After the recognition of the RNA:DNA junction, RNase H2 can incide the damage. On the contrary, the abasic rNMP is not bonded to the complementary strand, causing an orphan base on the complementary DNA strand. In this case, RNase H2 is not able to recognize the RNA:DNA junction and, consequently, cleave the abasic rNMP. We hypothesized that the lack of the hydrogen bonding between the abasic rNMP and the opposite deoxyribonucleotide interferes with the capacity of RNase H2 to recognize and cleave an abasic rNMP.

Differently from RNase H2, APE1 specifically recognizes and cleaves an abasic distortion in DNA, and engulfs the sugar-phosphate further distorting the DNA (Freudenthal et al. 2015b). We hypothesized that the ribose extra OH would have only minor influence on the structure, explaining how APE1 is able to cleave deoxy-abasic sites as well as ribo- abasic sites (Figure 40).

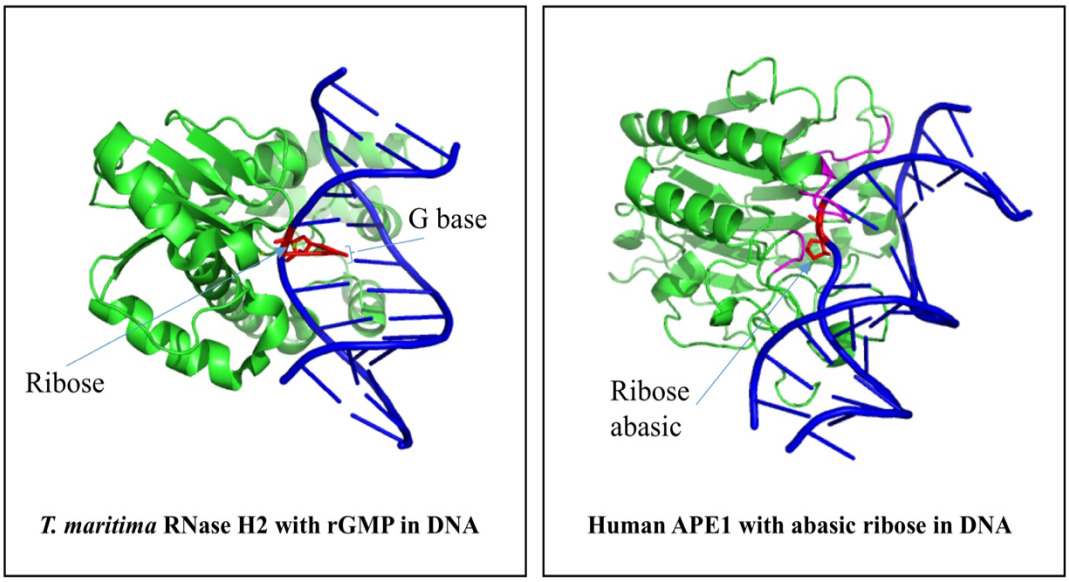

Figure 40 ***Structural models with the active site of RNase H2 and APE1 in presence of a rNMP or a rAP, respectively***

T. maritima (left) and human APE1 (right) is in a complex with DNA having a single rNMP or single abasic residue, respectively. DNA is indicated in blue, while the single rNMP and the abasic residue are shown in red as sticks. Ribose or G base or abasic sites are pointed by arrows. Proteins are shown in green. The part of APE1 that engulf the abasic sugar is colored in magenta. PDB for RNase H2 is 303-F; for APE1 as 1DEW (Courtesy of Professor Crouch-published in Malfatti et al., 2017).

In conclusion, we hypothesized a model in which 8oxo-ribonucleotides could be generated in the cellular nucleotide pool or even when they are already incorporated in DNA, possibly as a result of an oxidation of the sugar and/or the base.

After the evidence that human RNase H2 is not able to process an oxidized rNMP embedded in DNA, we found that APE1 shows a weak but significant activity on it. Similarly, RNase H2 does not process a rAP embedded in DNA, which could be generated spontaneously or by the r8oxoG processing, and again APE1 possesses a strong activity on this type of damage. Because APE1 activity on 8oxo-ribonucleotides in DNA is low, we hypothesize that other proteins (probably a unknown glycosylase) may participate in their repair (Figure 41).

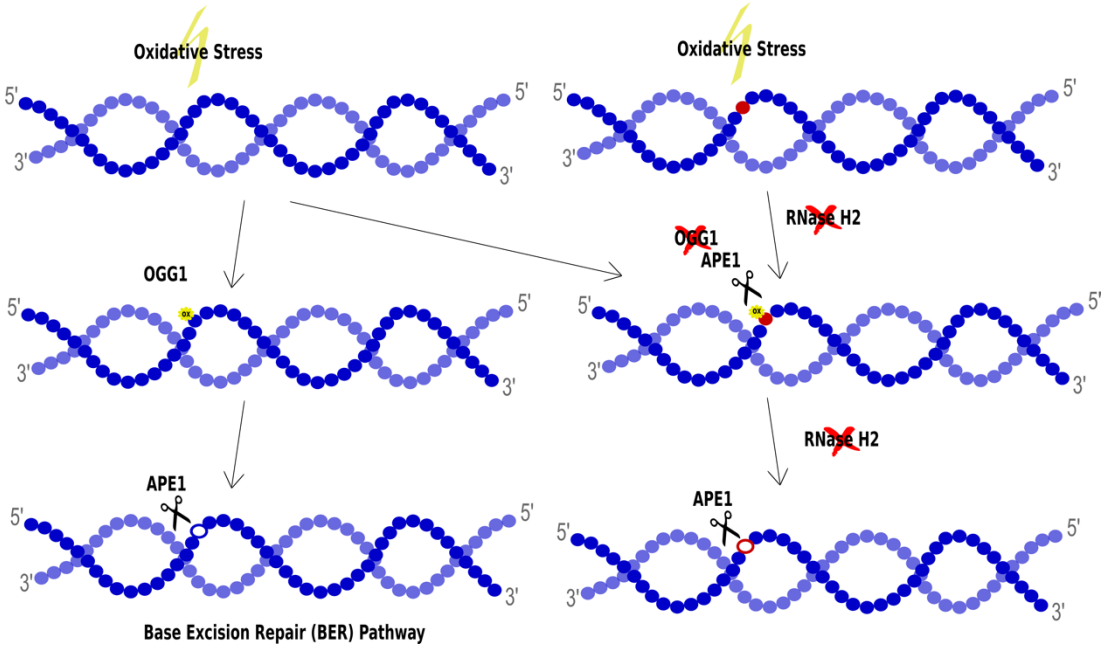

Figure 41 ***A model for the repair of oxidized and abasic rNMP sites embedded in DNA by APE1***

DNA (●), when subjected to oxidative stress (yellow flash of lightning) in which oxidized damaged bases are generated (yellow dot), is efficiently repaired by Base Excision Repair (BER) pathway in which OGG1, the main glycosylase, processes the oxidized base leaving a deoxy- abasic site (○) to APE1, the BER endonuclease, that cleaves the abasic site allowing the correct repair of damaged DNA (left side). At the same time, ribonucleotides embedded in DNA (●) can be oxidized following by oxidative stress. Although OGG1 is not able to process r8oxoG, APE1 shows a weak activity on this damaged rNMP. Probably, an unknown glycosylase might cleave r8oxoG generating a ribo-abasic site (○) that, as we have demonstrated, is efficiently processed by APE1 (right side).

RNase H2, the main enzyme of Ribonucleotide Excision Repair pathway, is not involved in the processing of both types of damaged rNMPs embedded in DNA.

9. Future Perspectives

Although important discoveries have been done in this direction, several unresolved questions still remain open.

As previously demonstrated in our lab, APE1-defective cells show increased oxidized rRNA content upon oxidative stress (Vascotto et al. 2009). Now, this observation seems to have a molecular explanation in the detected endoribonuclease activity of APE1 over r8oxoG- containing oligonucleotides and will deserve further attention in our future studies.

Confirming the inability of OGG1 in the processing of r8oxoG site in DNA, experiments are underway in order to address if other glycosylases may process oxidized rNMPs to generate rAP sites, which surprisingly are processed by APE1 in a very efficient way.

Thus, the first aim for the further development of this work is the identification of which enzyme(s) is recruited to cleave oxidized base of rNMPs incorporated in DNA generating an abasic rNMP site which may be subsequently processed by APE1.

For instance, a role for the YB-1 protein in recognizing oxidized ribonucleotides sites in RNA has also been hypothesized (Hayakawa et al. 2002), but no specific enzymatic mechanisms able to remove this oxidized base has been described, yet.

As mentioned before, the accumulation of the oxidized RNA upon silencing of APE1 expression, may be explained by the assumption that enzymatic removal of oxidized rNMPs may represent the limiting step in the process. In addition, since APE1 is overexpressed in different types of cancer, such as ovarian, gastro-esophageal, pancreatico-biliary, lung and breast cancers (Woo et al. 2014; Al-Attar et al. 2010), it would be interesting to determine whether any correlation exists between its expression level and presence of modified rNMPs in cancers. Additional studies are ongoing along this direction.

By starting from ribo-Seq analysis, we would like to re-adapt this technique in order to have the possibility to distinguish damaged, including

abasic and oxidized, rNMPs among the unmodified rNMPs embedded in DNA. Then, we will expand our knowledge about the formation of abasic ribonucleotides in DNA, that is still a matter of debate. Although, the existence of specific N-ribohydrolases, including the toxin ricin, has been already documented (Schramm 1997) to be able to generate abasic rNMPs in RNA molecules, besides spontaneous generation (Loeb and Preston 1986), more specific information are needed in this context.

Then, we will investigate how the mis-incorporation of undamaged rNMPs changes under oxidative stress and/or silencing of APE1.

Besides the weak direct activity of APE1 on r8oxoG, APE1 could be stimulated by a glycosylase activity allowing a faster turnover as demonstrated for DNA substrates (Vidal et al. 2001). Again, work is in progress along these lines to better inspect this mechanism and the putative glycosylase enzymes involved.

The activity of APE1 on r8oxoG and rAP sites embedded in DNA does not hide the possibility that a similar activity could be exerted on RNA molecules too. This finding could represent the first demonstration of an enzyme able to recognize and process oxidized RNA (Vascotto et al. 2009). Since RNA oxidation has been shown to exert detrimental physiological effects, and to be a common feature in different human pathologies ranging from aging to neurodegenerative and cancer diseases (Simms and Zaher 2016). This is testified by the fact that oxidized RNA (Rhee, Valentine, and Termini 1995) or RNA containing abasic sites (Kupfer and Leumann 2006) show inhibitory effects on reverse transcriptase activity, whereas oxidized mRNA (Shan, Chang, and Lin 2007; Tanaka, Chock, and Stadtman 2007) or mRNA with abasic sites (Hudak, Bauman, and Tumer 2002) exhibit compromised translation activity as well as translation fidelity (Calabretta, Kupfer, and Leumann 2015). Finding an enzyme involved in the processing of oxidized RNA could represent an amazing discovery in the DNA repair world that will open the way for the discovery of new molecular mechanisms underlying different pathogenesis and will set the basis for the creation of new drugs.

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"... It does not do to dwell on dreams and forget to live ..."

11. List of Publications (2014-2017)

- **"Inhibitors of the Apurinic/Apyrimidinic Endonuclease 1 (APE1)/Nucleophosmin (NPM1) Interaction That Display Anti-Tumor Properties",** Mattia Poletto, **Matilde Clarissa Malfatti,** Dorjbal Dorjsuren, Pasqualina L. Scognamiglio, Daniela Marasco, Carlo Vascotto, Ajit Jadhav, David J. Maloney, David M. Wilson III, Anton Simeonov, and Gianluca Tell, Molecular Carcinogenesis, 2016. 10.1002/mc.22313

- **"Unveiling the non-repair face of the Base Excision Repair pathway in RNA processing: A missing link between DNA repair and gene expression?",** Giulia Antoniali, **Matilde Clarissa Malfatti**, Gianluca Tell, DNA Repair, 2017. 10.1016/j.dnarep.2017.06.008

- **"Abasic and oxidized ribonucleotides embedded in DNA are processed by human APE1 and not by RNase H2", Matilde Clarissa Malfatti**, Sathya Balachander, Giulia Antoniali, Kyung Duk Koh, Christine Saint-Pierre, Didier Gasparutto, Hyongi Chon, Robert J. Crouch, Francesca Storici, and Gianluca Tell. 10.1093/nar/gkx723

- **"Human AP endonuclease activity on telomeric G4 structures is dependent on acetylatable Lysine residues on the N-terminal domain",** Silvia Burra, Daniela Marasco, **Matilde Clarissa Malfatti**, Giulia Antoniali, Bruce Demple, Antonella Virgilio, Veronica Esposito, Aldo Galeone, Gianluca Tell, *in preparation.*

- **"APE1/NPM1 axis in Triple Negative Breast Cancer", Matilde Clarissa Malfatti**, Lorenzo Gerratana, Carla Di Loreto, Fabio Puglisi, Gianluca Tell, *in preparation.*

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13. Published papers

This Thesis discusses the most part of experiments collected in the published paper entitled:

“Abasic and oxidized ribonucleotides embedded in DNA are processed by human APE1 and not by RNase H2”.

Following this, other two papers are in attachment, published during my PhD program, entitled:

“Inhibitors of the Apurinic/Apyrimidinic Endonuclease 1 (APE1)/Nucleophosmin (NPM1) Interaction That Display Anti-Tumor Properties”;

“Unveiling the non-repair face of the Base Excision Repair pathway in RNA processing: A missing link between DNA repair and gene expression?”.

Nucleic Acids Research, 2017 **1**
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Abasic and oxidized ribonucleotides embedded in DNA are processed by human APE1 and not by RNase H2

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ABSTRACT

Ribonucleoside 5′-monophosphates (rNMPs) are the most common non-standard nucleotides found in DNA of eukaryotic cells, with over 100 million rNMPs transiently incorporated in the mammalian genome per cell cycle. Human ribonuclease (RNase) H2 is the principal enzyme able to cleave rNMPs in DNA. Whether RNase H2 may process abasic or oxidized rNMPs incorporated in DNA is unknown. The base excision repair (BER) pathway is mainly responsible for repairing oxidized and abasic sites into DNA. Here we show that human RNase H2 is unable to process an abasic rNMP (rAP site) or a ribose 8oxoG (r8oxoG) site embedded in DNA. On the contrary, we found that recombinant purified human apurinic/apyrimidinic endonuclease-1 (APE1) and APE1 from human cell extracts efficiently process an rAP site in DNA and have weak endoribonuclease and 3′-exonuclease activities on r8oxoG substrate. Using biochemical assays, our results provide evidence of a human enzyme able to recognize and process abasic and oxidized ribonucleotides embedded in DNA.

INTRODUCTION

Incorporation of ribonucleotides monophosphate (rNMPs) in DNA is a frequent phenomenon, which is considered the most common type of 'DNA damage' occurring in normal cells (1,2). Ribose-seq and other approaches recently developed for mapping sites of rNMPs in DNA have shown widespread but not random distribution of rNMPs in chromosomal DNA of budding and fission yeast (2) (and references therein). The number of rNMPs identified per nuclear chromosome was found to be proportional to chromosome size (2), and quantitation approaches have estimated a 600,000 rNMPs in budding yeast genome and over 100 millions in mouse genome (3).

The incorporation of rNMPs in genomic DNA may be due to: (i) the disequilibrium in the cellular pool of deoxyribonucleotides (dNTPs) and ribonucleotides (rNTPs) (2), (ii) an incomplete elimination of RNA primers used in the generation of Okazaki fragments (4), (iii) an oxidation of the deoxyribose sugar into ribose (5) and last but not least, (iv) an imprecise 3′-exonucleolytic proofreading activity of replicative DNA polymerases, which do not discriminate rNMPs from dNMPs pool (2,4,6,7). Furthermore, taking into consideration all rNMPs that are synthesized during lagging strand synthesis, more than 100 million rNMPs are introduced into the mammalian genome per cell cycle (3). In addition, it has been estimated that the amount of rNTPs is generally 40–350-fold higher than that of dNTPs in cycling cells (6–8) increasing the probability of incorrect rNMP incorporation during DNA replication and repair (2,4,6,7).

The effects of the 2′-hydroxyl group of the ribose sugar within an rNMP embedded in DNA are numerous: it mainly alters DNA elasticity and structure in a sequence dependent manner (9–11) and affects the activity and function of several DNA-interacting proteins, in addition to increase the DNA fragility and mutability (1,12). Moreover, rNMPs in DNA can be template for DNA synthesis (13,14), although the DNA polymerases processivity on rNMP tracts is reduced (13).

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The main pathway deputed to rNMPs removal is ribonucleotide excision repair (RER), in which the principal enzyme is RNase H2. It was demonstrated that RNase H2 deficiency in mammalian cells is associated to DNA damage repair activation and, in humans, to pathology. RNase H2-null murine embryonic fibroblasts (MEFs) activate a p53-dependent damage response, whereas null-RNase H2 are embryonic lethal (15). In humans, mutations in each of the three subunits of RNase H2 are associated with the neurological syndrome of Aicardi-Goutières (AGS), which causes severe brain dysfunction (16–18). Altered RNase H2 function in AGS patients may result in increased level of rNMPs in DNA, which could in turn activate DNA damage response signaling and induce innate immune response (19,20).

Besides RER, other different DNA repair mechanisms are active to remove rNMPs embedded in DNA (3,21). In the absence of RNase H2, topoisomerase I cleavage (21), followed by nick processing by Srs2–Exo1, can remove some rNMPs (22,23). rNMPs in DNA can be also targeted by the nucleotide excision repair (NER) factors in bacteria (24). However, *in vitro* experiments showed that human NER proteins are not active to remove rNMPs embedded in DNA (25). Differently, the mismatch repair (MMR) mechanism targets mismatches with rNMPs both in *Escherichia coli* and *Saccharomyces cerevisiae* genomic DNA (26). Until now, there is no proof that the base excision repair (BER) mechanism plays any role in removing rNMPs from DNA.

BER is known to repair a wide spectrum of oxidative lesions in nuclear and mitochondrial DNA (27,28), and preventing cancer (29,30). Abasic sites, which form by spontaneous hydrolysis of the N-glycosidic bond in DNA or following removal of a damaged base by BER glycosylases, are major targets or intermediate substrates in the BER pathway of DNA repair (31). It has been estimated that up to 10,000 abasic sites are formed per human genome per day (32). Despite the fact that spontaneous depurination occurs ∼1,000 times slower in RNA than DNA (33), due to the high abundance of rNMPs in genomes, with >100 million rNMPs transiently present in mammalian DNA during one replication cycle, considering rNMP incorporation by DNA polymerases during DNA replication and repair, and RNA primers of Okazaki fragments (3), the possibility that abasic and oxidized rNMPs (such as 7,8-dihydro-8-oxoriboguanosine) are present in DNA and are targets of BER is quite real and worth careful study. These data, together with other recent findings about the ability of *Schizosaccharomyces pombe* Pol 4, *Mycobacterium smegmatis* DinB2 and human Polβ to insert and elongate oxidized rGMP when paired with dA during DNA replication (34–36), underscore the necessity to determine how cells can target and remove oxidized rNMPs or rAP sites from DNA.

Because recent studies point toward a new function of BER in RNA surveillance (37,38), there is high likelihood that BER could be involved in the processing of rNMPs in DNA, particularly in the case of chemically modified rNMPs, such as abasic and oxidized rNMPs. Identifying whether BER may target normal and modified rNMPs in DNA is important to better understanding the mechanism of genotoxicity of reactive oxygen species, the function and the impact of BER defects in human disease and cancer mechanisms. In the absence of proper repair mechanisms to cope with these kind of lesions, even a single or a few modified rNMPs present in a genome per cell cycle could lead to mutations and/or genomic rearrangements.

Findings from our and other laboratories have revealed an important involvement of the apurinic/apyrimidinic endonuclease 1 (APE1) in RNA metabolism and RNA-decay (37–40). APE1 is by far one of the most studied enzymes in the BER pathway for its altered expression in different human pathologies ranging from neurodegenerative to cancer disorders (41). Its role in DNA repair is primarily due to its ability to act as an endonuclease, specifically able to cleave 5′ to deoxy- abasic sites, which results in a strand break with 3′-hydroxyl and 5′-phosphodeoxyribose termini. APE1 also has redox activity needed to modulate the DNA binding ability of several transcription factors (41). As recently demonstrated, APE1 can endonucleolytically cleave abasic single-stranded RNA (37,38,40), has a 3′-RNA phosphatase activity, and a weak 3′-5′ exoribonuclease activity (42). Moreover, it has been demonstrated that APE1 has nucleotide incision repair (NIR) activity on modified bases, such as 5,6-dihydro-2′-deoxyuridine, 5,6-dihydrothymidine, 5-hydroxy-2′-deoxyuridine, 5-hydroxycytosine, which are directly repaired by APE1 bypassing the action of specific glycosylases (43–45). Therefore, we hypothesized that APE1 can be involved in processing rNMPs in DNA, particularly in the case of abasic and oxidized rNMPs.

Here, we found that eukaryotic RNase H2 from yeast, mouse and human is inactive on an rAP site in DNA in different assays. We discovered and characterized an unknown APE1 activity on abasic ribonucleotide embedded in DNA. We then compared the ability of human RNase H2 to cleave at an oxidized ribonucleotide (r8oxoG) incorporated in a DNA substrate and analyzed the activities of 8-oxoguanine DNA glycosylase (OGG1) and APE1 to recognize and cleave this particular type of damage. Our data demonstrate that APE1, but not human RNase H2 and OGG1, has a weak endoribonuclease activity on the oxidized substrate.

MATERIALS AND METHODS

Double strand synthetic oligonucleotides description and annealing conditions

All oligonucleotides and their complementary sequences used in this study are listed in Supplementary Table S1 (see also Supplementary Figure S1). ss_dG_40 oligonucleotide and its reverse complementary sequence, ss_dC_40, were purchased from Invitrogen (Grand Island, NY, USA). ss_rG_40 oligonucleotide and the DNA oligo containing a tetrahydrofuran abasic deoxyribonucleotide, ss_dF_40, or tetrahydrofuran abasic ribonucleotide, ss_rF_40, as well as ribo- 1′OH abasic containing oligonucleotide, ss_rOH, were purchased from Dharmacon (GE Healthcare, Lafayette, CO, USA). The 26-mer oligonucleotide containing a tetrahydrofuran, ss_dF, and its reverse complementary sequence ss_dC, were synthesized from Metabion International AG (Steinkirchen, Germany). The 25-mer dG-, rG-, d8oxoG-containing oligonucleotides and complementary oligonucleotides were synthesized from Metabion International AG (Steinkirchen, Germany).

ss_dG_40, ss_rG_40, ss_dF_40 and ss_rF_40 oligonucleotides were 5′ end-labeled with [γ-^{32}P] ATP (PerkinElmer, Boston, MA, USA) by T4 polynucleotide kinase (PNK) (New England BioLabs, Ipswich, MA, USA) in a reaction mixture containing 10 μM ATP using 10X PNK buffer (New England BioLabs, Ipswich, MA, USA). This labeling reaction was incubated at 37°C for 1 h, followed by inactivation at 65°C for 20 min. The reactions were purified by using Illustra MicroSpin G-25 column (GE Healthcare, Buckinghamshire, UK).

The remaining oligonucleotides were labelled with either IRDye700, IRDye800 fluorophores or Cyanine5 at 5′ end, as specified in Supplementary Table S1, purified through RP-HPLC, checked in Mass Check and re-suspended in RNase- and DNase- free water.

Synthesis of oligonucleotide containing an internal ribose 8-oxo-guanosine (r8oxoG) and an IRDye700 fluorophore at 5′ end was in-house carried out on an Applied Biosystems 392 DNA/RNA synthesizer using the phosphoramidite chemistry, associated with the phenoxyacetyl protecting group for the nucleobases and the *tertio*-butyldimethylsilyle protecting group at the 2′-OH position of the ribonucleoside residue (46). Upon completion, the oligonucleotide was de-protected in concentrated aqueous ammonia for 6 h at 55°C, followed by a desilylation step with triethylamine trihydrofluoride (8 h at room temperature) (46) and was finally purified by preparative 20% denaturing PAGE using UV-shadowing detection. After desalting by size exclusion, the r8oxoG oligonucleotide was quantified by UV measurements at 260 nm and its purity was checked by RP-HPLC analysis together with MALDI-TOF mass measurements (Supplementary Figure S5, panels A and B). Sample was then lyophilized and frozen at –20°C until use.

All oligonucleotides used in the present study were re-suspended in RNase- and DNase-free water at 100 μM. 100 pmol of each oligonucleotide was annealed with an excess of 150 pmol of its complementary DNA oligonucleotide (as indicated in Supplementary Table S1) in 10 mM Tris–HCl pH 7.4 and 10 mM $MgCl_2$, heated at 95°C and cooling down over night in the dark.

Plasmid and expression of recombinant proteins

Plasmids and expression of human recombinant OGG1 enzyme was purified as described by Audebert *et al*. (47). Plasmids and expression of human recombinant APE1 wild type (WT) and respective mutants (APE1 NΔ33 and APE1 E96A) were produced as explained by Fantini *et al.* (48) and Erzberger and Wilson (49). Plasmid and expression of yeast, mouse and human recombinant RNase H2 were produced as explained by Chon *et al.* (50,51).

Cell lines and silencing experiments

HeLa cells (human cervical carcinoma) (ATCC®, Milan, Italy) were grown in DMEM (EuroClone, Milan, Italy) supplemented with 10% fetal bovin serum (FBS-EuroClone, Milan, Italy), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) (EuroClone, Milan, Italy) and cultured in a humidified incubator at 5% CO_2 at 37°C.

For silencing experiments, 15 × 10^4 cells were seeded and transfected with 5′ UACUCCAGUCGUACCAGACCU 3′ siAPE1 (100 pmol) or siGENOME SMART pool siRNase H2A (50 pmol) or 5′ CCA UGA GGU CAG CAU GGU CUG UU 3′ scramble control siRNA (100 pmol) (GE Dharmacon, Milan, Italy) by using Oligofectamine™ Reagent (GE Dharmacon, Milan, Italy) as per manufacturer's indications. After 72 h upon transfection, cells were harvested by trypsinization and centrifuged at 250 × g for 5 min at 4°C. Supernatant was removed, and pellet was washed once with ice-cold phosphate-buffered saline without Calcium and Magnesium (PBS-Euroclone, Milan, Italy) and then centrifuged again (250 × g for 5 min at 4°C).

Preparation of nuclear cell extracts (NCE)

After washing with PBS, cells were collected in cold PBS added with 0.1 M DTT and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were centrifuged at 800 × g for 10 min at 4°C and the supernatant was removed. Pellet was re-suspended in a cold hypotonic solution containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA pH 8.0 complemented with 0.1 mM DTT, 0.5 mM PMSF, 1 mM protease inhibitor (PI), 1 mM NaF, 1 mM Na_3VO_4. After centrifugation at 800 × g for 10 min at 4°C, cytosolic proteins (CCE) were collected whereas intact nuclei were pelleted. Pellet was washed to discard any contamination from cytosol and it was subsequently re-suspended with a cold hypertonic solution 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA pH 8.0, 5% glycerol complemented with 0.1 mM DTT, 0.5 mM PMSF, 1 mM PI, 1 mM NaF, 1 mM Na_3VO_4 and incubated on ice for 30 min. At the end, the sample was centrifuged at 15,000 × g for 20 min at 4°C and collected the supernatant containing nuclear proteins (NCE). Quantification of each sample was performed by colorimetric Bradford assays (Bio-Rad, Milan, Italy).

Preparation of whole cell extracts (WCE)

After washing with PBS, cells were harvested by trypsinization and centrifuged at 250 × g for 5 min at 4°C. The supernatant was removed, washed once with PBS and centrifuged again. Pellet was re-suspended in a lysis solution containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (w/v) Triton X-100 supplemented with 1 mM PI, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4. After centrifugation at 15 000 × g for 20 min at 4°C, the supernatant is considered as whole cell extract (WCE). Proteins of each sample were quantified using a colorimetric Bradford assays (Bio-Rad, Milan, Italy).

Enzymatic activity assays

To measure enzymatic activity of recombinant proteins and NCE on different substrates, each reaction was prepared following doses, time points and buffers specified in detail into the legend of each experiment. Final volume for each reaction was 10 μl. At the end of all reactions, samples were blocked with a stop solution, containing 99.5% (v/v) formamide (Sigma-Aldrich, Milan, Italy) supplemented with

10× Orange Loading Dye (Li-Cor Biosciences, Milan, Italy) and heated at 95°C for 5 min. Then, all samples were loaded onto a 7 M denaturing 20% polyacrylamide gel in TBE buffer pH 8.0 and run at 4°C at 300 V for 1 h. Then, the gel was visualized with an Odyssey CLx Infrared Imaging system (LI-COR GmbH, Germany). The signals of the non-incised substrate (S) and the incision product (P) bands were quantified using Image Studio software (LI-COR GmbH, Germany). When using the ds_rOH:dC and ds_r8oxoG:dC oligonucleotides, a very small amount of cleavage product was seen in samples not treated with recombinant proteins and/or cell extracts due to the reactivity of this molecule, which was spontaneously degraded. During the analysis, this band has been always subtracted from bands obtained following treatment with recombinant proteins and/or extracts.

For radioactive experiments, reactions were stopped by adding 2× denaturing PAGE gel buffer (0.01% bromophenol blue, 95% formamide and 20 mM EDTA pH 8.0) and heating to 95°C for 5 min. After dilutions, the products were analyzed by 15% (w/v) polyacrylamide, 8 M urea gel electrophoresis (urea–PAGE). 20–100 Oligonucleotide Length Standard (Integrated Device Technology, Coralville, IA, USA) was used as a ladder (M). After electrophoresis, gels were exposed to phosphor screen overnight. Images were taken with Typhoon Trio+ (GE Healthcare, Lafayette, CO, USA) and obtained with ImageQuant (GE Healthcare). Band intensities were quantified by Multi Gauge V3.0 (Fujifilm).

Electrophoretic mobility shift assay analysis (EMSA)

Proteins binding to nucleic acids was assessed by EMSA analysis as already described by Fantini *et al*. (48). Briefly, the indicated amounts of recombinant purified proteins or cell extracts were co-incubated with 250 fmol of the probe (25 nM) at 37°C for 30 min. Reactions were prepared in a buffer containing 8 mM HEPES, 10 mM KCl, 400 μM EDTA pH 8.0, 5 mM DTT and 2% glycerol in a 10 μl final volume. Moreover, salmon sperm DNA (SSD) (Sigma-Aldrich, Milan, Italy) was added like as DNA competitor. Samples were loaded onto an 8% (w/v) native polyacrylamide gel in tris-sodium acetate–EDTA pH 8.0 (TAE) buffer and run at 4°C at 150 V for 1 h followed by 3 hat 250 V.

Statistical analysis

Statistical analyses were performed by using the Student's *t* test. $P < 0.05$ was considered as statistically significant.

RESULTS

Human, yeast or mouse RNase H2 does not process an rAP site embedded in DNA

RNase H2 is the principal protein able to process paired and mismatched rNMP sites embedded in DNA by generating a nick to their 5′ side (26) (and references therein). Up to now, whether RNase H2 can cleave an rAP site incorporated in a duplex DNA is unknown. In order to test this hypothesis, we measured RNase H2 ability to cleave a modified

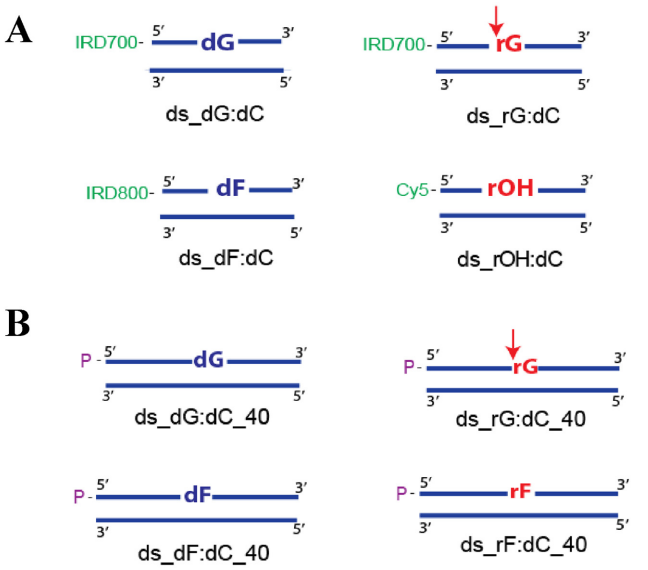

Figure 1. Scheme of substrates used to test cleavage of an rAP site in DNA. DNA nucleotides are in blue, RNA in red. The 5′ and 3′ ends of each DNA strand are indicated. (**A**) Scheme of double strand (ds) DNA 25-mer substrates (single strand (ss) oligonucleotide annealed to ss_dC containing-complementary oligonucleotide) containing a dGMP, rGMP, dF and 1′ OH abasic rNMP (rOH) site in the 13th position. The 'IRD700' in green indicate IRDye 700 phosphoramidite dye tagged at the 5′ end. The 'IRD800' in green indicate IRDye 800 phosphoramidite dye tagged at the 5′ end. The 'Cy5' in green indicates cyanine dye tagged at the 5′-end of the top strand of the duplex. (**B**) Scheme of the ds_DNA 40-mer substrate (ss oligonucleotide annealed to its ss complementary oligonucleotide) containing a dGMP, rGMP, dF and rF site in the 20th position. The P in purple indicates radiolabelled ^{32}P at the 5′-end of the top strand of the duplex. The red arrow indicates the cleavage position by RNase H2 5′ to the rGMP site.

25-mer DNA oligonucleotide, called ds_rOH:dC, in which a 1′-OH abasic rNMP was incorporated into a DNA substrate as shown in Figure 1A (see also Supplementary Table S1 and Supplementary Figure S1). First of all, recombinant human RNase H2, composed of its three subunits, was purified as explained in Materials and Methods (Supplementary Figure S2) and its activity was tested on ds_rOH:dC oligonucleotide in parallel with dG- and rG- containing oligonucleotides as negative and positive controls, respectively. As reported in Figure 2, the enzyme had no activity on ds_dG:dC and ds_dF:dC (containing a tetrahydrofuran residue mimicking the abasic site) oligonucleotides, whereas it efficiently cleaved the canonical rG substrate as expected. In addition, we tested the mismatched rG- containing oligonucleotide, ds_rG:dA, confirming that RNase H2 protein is also active on this type of substrate (Figure 2A). On the other hand, no activity was detectable on ds_rOH:dC demonstrating that recombinant human RNase H2 is not able to process an abasic rNMP embedded in DNA. To confirm these data in cells, we tested the activity of RNase H2 protein obtained from nuclear cell extracts on the same substrates, as above (Figure 2). To this aim, RNase H2 expression was downregulated in HeLa cells through specific siRNA and the endoribonuclease activities of nuclear extracts from knocked down and control cells were then assayed. Western blotting analyses performed on nuclear extracts from control (Scramble) and knocked down

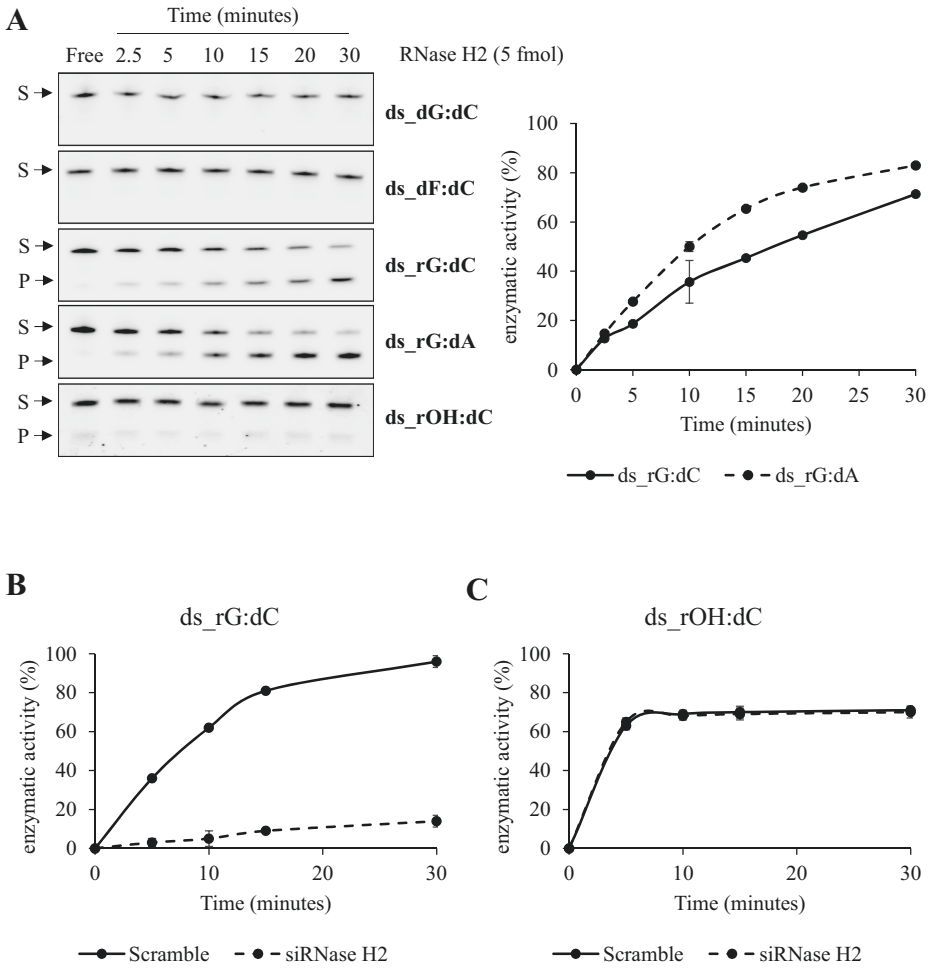

Figure 2. Human RNase H2 is not able to process an rAP site embedded in a duplex DNA substrate. (**A**) Representative denaturing polyacrylamide gel of oligonucleotides (25 nM) incision by recombinant human RNase H2 (0.5 nM). The reaction was performed in RNase H2-buffer (20 mM Tris–HCl, 25 mM KCl, 0.1% BSA, 0.01% Tween20, 4 mM $MgCl_2$, pH 7.4) for different time points, expressed in minutes and shown on the top of the figure, at 37°C. ds_dG:dC and ds_dF:dC oligonucleotides were used as negative controls whereas paired and mismatched ds_rG oligonucleotides as positive controls. S indicates the substrate position while P indicates the product position (*left*). Relative graph illustrating the time-course kinetics activity of the recombinant protein on ds_rG:dC and ds_rG:dA oligonucleotides. Data are expressed as mean ± SD of three independent technical replicas (*right*). (**B**) Graph illustrating the time-course kinetics activity of NCE on ds_rG:dC in control and RNase H2-knocked down conditions. Enzymatic reaction was performed at 37°C in RNase H2-buffer with 500 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always less than 10% of the mean of the experimental points. (**C**) Graph illustrating the time-course kinetics activity of NCE on ds_rOH:dC oligonucleotide in control and RNase H2-knocked down conditions. Enzymatic reaction was performed at 37°C in RNase H2-buffer with 500 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always less than 10% of the mean of the experimental points.

(siRNase H2) cells demonstrated the efficiency of RNase H2 downregulation (∼50%) upon transfection with specific siRNA sequences (Supplementary Figure S3A). We incubated Scramble or siRNase H2 cell extracts with different substrates for the indicated time points. Following knock-down of RNase H2, we found a decreased cleavage of ds_rG:dC, as expected (Figure 2B and Supplementary Figure S3B). Surprisingly, we found that ds_rOH:dC was also cleaved; however, ds_rOH:dC cleavage was completely unaffected by siRNase H2 cell extracts (Figure 2C and Supplementary Figure S3B). These data suggest that while human RNase H2 is inactive on an abasic rNMP embedded in DNA, there is another enzyme/s capable of cleaving it in human cells.

To increase the stability of the abasic rNMP-containing oligonucleotide, we also used abasic substrates mimicked by tetrahydrofuran (F) residues, similarly to what commonly used for dNMP (52,53). We then tested whether RNase H2 from yeast or mouse may process an abasic rNMP, mimicked by a tetrahydrofuran ribonucleotide residue, embedded in a longer DNA sequence composed of 40-bp to evaluate a possible role of the length of the substrate in determining the inability of RNase H2 to process these substrates (Figure 1B). We used single-stranded (ss) or double-stranded (ds) DNA substrates containing an abasic rNMP site (rF), an rGMP (rG), an abasic dNMP (dF) or a dGMP as internal controls (Supplementary Table S1). As expected, *S. cerevisiae* and mouse RNase H2 cleaved at the single rG in a DNA duplex substrate (*lane 9* in Figure 3, panels A and B, respectively) and had no activity on the rG embedded in the ss substrate (*lanes 7* and *8* in Figure 3, panels A and B). Importantly, as we found for human RNase H2, also *S.*

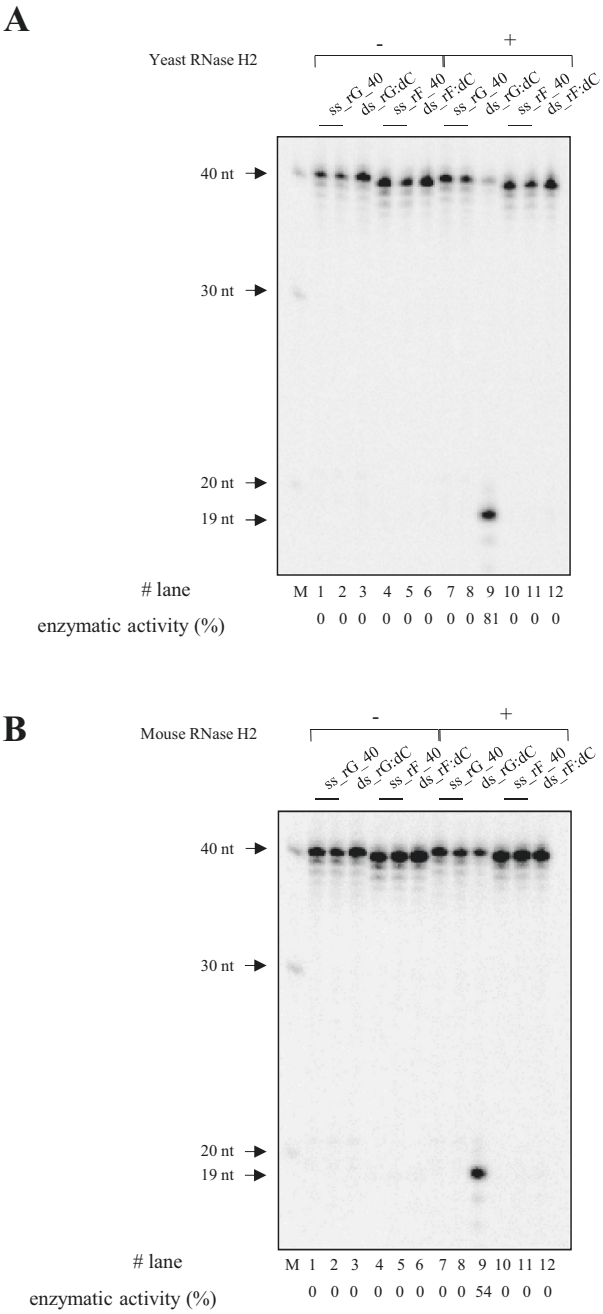

Figure 3. Mouse and yeast RNase H2 are not able to process an rAP site embedded in a duplex DNA substrate. (A and B) Denaturing PAGE gels showing cleavage result using 10 nM of 40-mer radioactive substrate containing an rG or an rF site, without (*lanes 1–6*) or with (*lanes 7–12*) 10 nM of yeast RNase H2 protein (**A**) or 10 nM of mouse RNase H2 protein (**B**). All reactions were carried out at 37°C for 1 h in yeast/mouse reaction 1× buffer (15 mM Tris–HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT and 0.1 mg/mL BSA). M indicates the DNA ladder and the black arrows on the left of each panel show specific band sizes. *Lanes 1,7* have ss-substrate containing rG (ss_rG_40), *lanes 2,8* have ss-substrate containing ss_rG_40 that is cooled slowly at room temperature to demonstrate the absence of any self-annealing structures, and *lanes 3,9* show the ds-substrate containing rG (ds_rG_dC). *Lanes 4,10* have ss-substrate containing abasic ribo site (ss_rF_40), *lanes 5,11* have ss_rF_40 that is cooled slowly in room temperature to observe any self-annealing; and *lanes 6,12* have ds substrate containing abasic ribo site (ds_rF:dC). The percentages of cleavage of each reaction are displayed below the images as enzymatic activity (%).

cerevisiae and mouse RNase H2 complexes were inactive on the abasic rNMP, ds_rF:dC (*lane 12* in Figure 3, panels A and B). Together these results demonstrate the inability of eukaryotic RNase H2, both from yeast and mammalian origins, to process an abasic rNMP incorporated in DNA independently from the nature of the abasic site (either 1′-OH or tetrahydrofuran residue) and the length of the substrate (either 25- or 40-mers).

Human APE1 is able to process an rAP site embedded in DNA through its endonuclease catalytic domain

To test the ability of APE1 to process rAP sites in DNA, we purified human recombinant APE1 protein, as described in Materials and Methods section (Supplementary Figure S4A). The endonuclease activity of APE1 on ds_rOH:dC substrate was examined through cleavage assays. As a positive control for APE1 endonuclease activity, an oligonucleotide substrate containing a tetrahydrofuran residue mimicking the abasic site, called ds_dF:dC (52,53), was used. As reported in Figure 4A, and measured through kinetics experiments in Table 1, APE1 processes the abasic rNMP as efficiently as the canonical deoxyabasic site having a lower affinity for the ds_rOH:dC than the ds_dF:dC (11-fold increase of the K_M) but a higher catalytic rate (27-fold increase in the k_{cat}/K_M ratio) (Table 1). Moreover, APE1 was unable to process the rG- containing oligonucleotide, which is the preferential substrate of RNase H2 enzyme. To further characterize the enzymatic activity of APE1, we used the purified recombinant mutant APE1 E96A protein, in which the missense mutation of the residue in the catalytic site, characterized by the substitution of the glutamic acid in position 96 with alanine, causes a decreased enzymatic activity of the protein, lacking the ability to coordinate the Mg^{2+} ion in the catalytic site (52,54,55) (Supplementary Figure S4B). In addition, we used the purified recombinant mutant APE1 NΔ33 protein, in which the first 33 N-terminal residues, responsible for RNA-protein interaction but not affecting its enzymatic activity, have been deleted (32,38) (Supplementary Figure S4B). Following incubation of ds_rOH:dC with APE1 E96A mutant, there was barely any endonuclease activity, whereas the activity of APE1 NΔ33 mutant was comparable with that of the APE1 WT protein (Figure 4, panels B and C and Supplementary Figure S4C). These data demonstrate that the catalytic domain of APE1 is responsible for recognizing and cleaving a rAP site in dsDNA and that the N-terminal domain does not play any major role in the enzymatic activity on this substrate and that AP endonucleolytic activity on rAP sites is intrinsic to the purified protein.

Kinetic parameters (K_M, V_{MAX} and k_{CAT}) were calculated from the measurement of the endonucleolytic reaction rates for APE1 on ds_dF:dC and ds_rOH:dC substrates. As described by Fantini *et al.* (48), increasing concentrations of the substrate were incubated with a selecting concentration of the protein (see first column) in a time-course experiment. Kinetic values were calculated using a Lineweaver–Burk plot analysis and represent the mean ± SD of three independent experiments.

To confirm that APE1 is the major enzyme capable of cleaving ribo-abasic containing sites in cells, we used

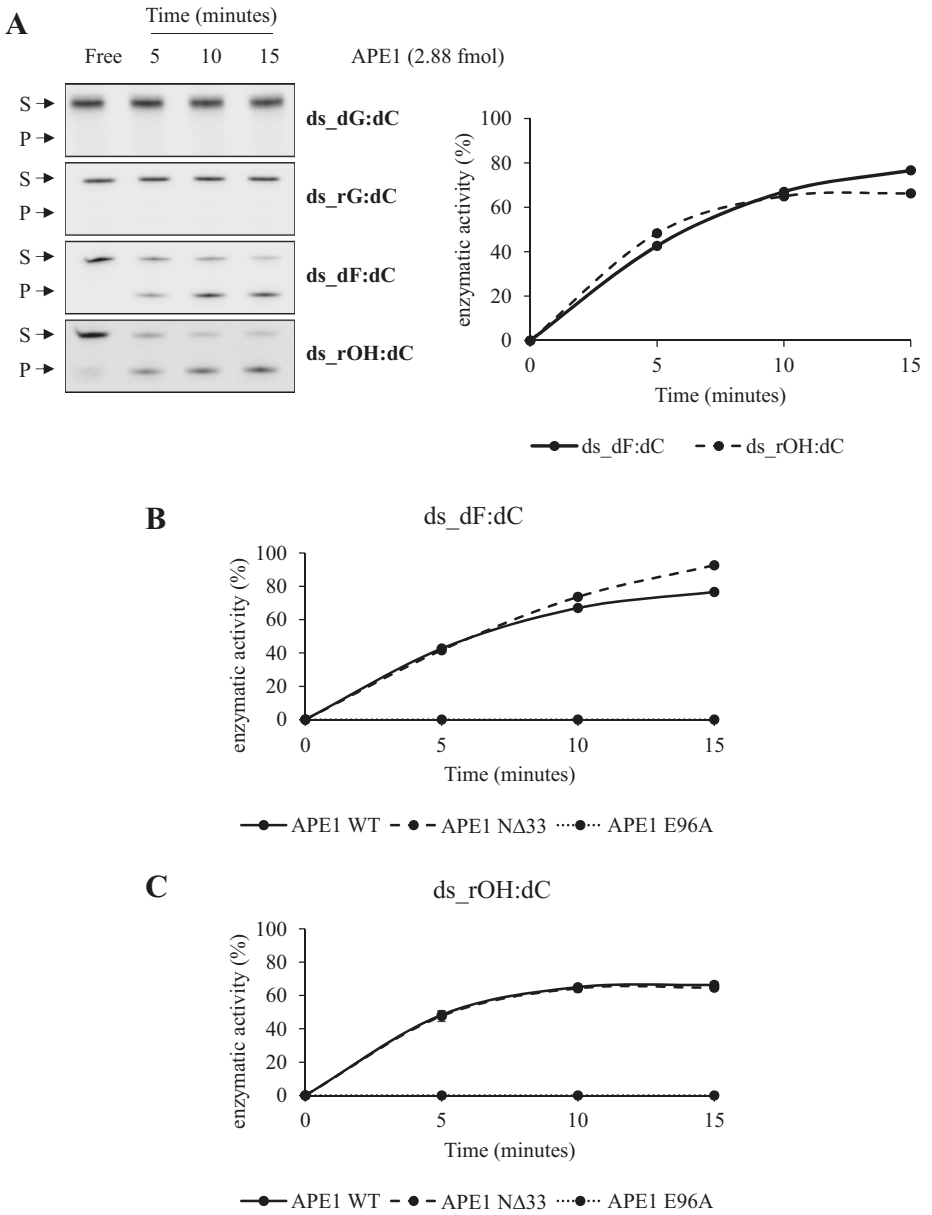

Figure 4. Human APE1 efficiently processes an rAP site embedded in a duplex DNA substrate. (**A**) Representative denaturing polyacrylamide gel of oligonucleotides (0.25 μM) incision by recombinant human APE1 (0.288 nM). The reaction was performed in APE1-buffer (20 mM Tris–HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4) for different time points, expressed in minutes and shown on the top of the figure, at 37°C. ds_dG:dC and ds_rG:dC oligonucleotides were used as negative controls, whereas ds_dF:dC oligonucleotide as positive control. S indicates the substrate position, while P indicates the product position (*left*). Relative graph illustrating the time-course kinetics activity of the recombinant protein on ds_dF:dC and ds_rOH:dC oligonucleotides. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always <10% of the mean of the experimental points (*right*). (**B**) Graph illustrating the time-course kinetics activity of APE1 mutants on ds_dF:dC oligonucleotide. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always <10% of the mean of the experimental points. (**C**) Graph illustrating the time-course kinetics activity of APE1 mutants on ds_rOH:dC oligonucleotide. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always <10% of the mean of the experimental points.

Table 1. Kinetic parameters for APE1 endonuclease activity on different substrates

[APE1] ($\times 10^{-3}$ nM)	Substrate	K_M (nM)	V_{MAX} (nM/min)	k_{CAT} (min^{-1})	k_{CAT}/K_M $(min.nM)^{-1}$
75	ds_dF:dC	14.2±6.98	0.95±0.33	12.7±4.39	0.95±0.16
3.125	ds_rOH:dC	158±41.79	12.66±3.71	4054±1191.8	26±1.96

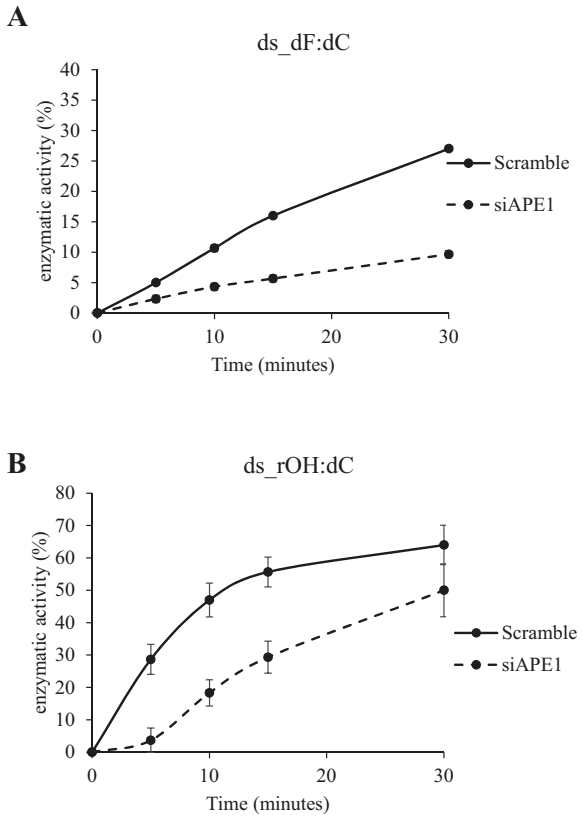

Figure 5. APE1 knock down in human cells impairs the processing of an rAP site embedded in a duplex DNA substrate. (**A**) Graph illustrating the time-course kinetics activity of NCE on ds_dF:dC in control and APE1-knocked down conditions. Data are expressed as mean ± SD of three independent technical replicas. Standard deviation values were always less than 10% of the mean of the experimental points. (**B**) Graph illustrating the time-course kinetics activity of NCE on ds_rOH:dC oligonucleotide in control and APE1-knocked down conditions. Enzymatic reaction was performed at 37°C in APE1-buffer with 10 ng of NCE. Data are expressed as mean ± SD of three independent technical replicas.

nuclear cell extracts in which APE1 was knocked-down through specific siRNAs (Supplementary Figure S3A), as explained in Materials and Methods. The endonuclease activity of APE1-kd (siAPE1) cell extracts, was reduced on both ds_dF:dC (Figure 5A and Supplementary S4D) and ds_rOH:dC (Figure 5B and Supplementary S4D), as compared to its respective control SCR-treated extracts. As western blot analysis shows (Supplementary Figure S3A), the expression of APE1 protein did not exert any effect on the expression of RNase H2 protein itself, demonstrating that the observed reduction of the processing activity of the abasic rNMP-containing substrate, observed with APE1-kd cell extracts, was likely due to the reduced expression of the APE1 protein. We further examined the specificity of the enzymatic activity of APE1 using the tetrahydrofuran ribonucleotide mimicking an abasic residue. We tested the activity of the APE1 E96A mutant to cleave at a ds_rF:dC substrate compared to that of APE1 WT (Figure 6A). As a control, the activity on the ds_dF:dC substrate was also analyzed. As it can be observed, mutant E96A showed a reduced cleavage on the ds_rF:dC substrate. Indeed, APE1 WT gave a 96% of cleavage at 5 and 10 nM (*lanes 2 and 3*), whereas mutant APE1 E96A had 32% cleavage at 5 nM and 38% at 10 nM (*lanes 5 and 6*). Cleavage of ds_rF:dC by APE1 WT was 89% at 5 nM and 90% at 10 nM (*lanes 8 and 9*), and there was minimal activity for APE1 E96A on the ds_rF:dC substrate in the same conditions (*lanes 11 and 12*). Moreover, pre-treatment of cell extracts with 0.2 nM of Compound #3 (i.e. *N*-(3-(benzo[d]thiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl)acetamide)), a specific APE1 endonuclease inhibitor (53,56), exerted a significant inhibitory effect upon APE1 enzymatic activity on both ds_dF:dC in DNA and ds_rF:dC substrates demonstrating that the main enzymatic activity of cell extracts was due to APE1 function (Figure 6, panels B and C).These data demonstrate that human APE1 is the major enzyme capable of specifically cleaving at abasic rNMPs in DNA while being unable to process normal rNMPs, which are the preferential substrates of RNase H2.

Human RNase H2 does not process an r8oxoG embedded in a duplex DNA

An abasic site can be generated spontaneously or following the processing of an oxidized lesion from a specific glycosylase. While known glycosylases (such as OGG1) are responsible for this activity on 8oxo-dG, no enzyme is known to be able to process the oxidized rG substrate. We then focused our attention on r8oxoG removal. The r8oxoG containing oligonucleotide was in-house synthesized and, as observed from MALDI-MS analysis and HPLC purification (Supplementary Figure S5, panels A and B), the undesirable presence of secondary products of the chemical synthesis of this substrate can be excluded. First of all, we investigated whether human RNase H2 was able to recognize and cleave at r8oxoG site using an oligonucleotide containing this type of lesion called ds_r8oxoG:dC (Figure 7A, see also Supplementary Table S1, Supplementary Figure S1). In these experiments, we always compared the specific enzymatic activity of RNase H2 with that exerted on the canonical ds_rG:dC substrate, as positive control, and using the ds_d8oxoG:dC oligonucleotide as negative control. As Figure 7B shows, while the ds_rG:dC oligonucleotide was efficiently processed by RNase H2, the same activity was not observed for ds_r8oxoG:dC. As expected, the d8oxoG-containing oligonucleotide was not cleaved by RNase H2. These data confirm that RNase H2 was not able to process modified rNMPs embedded in DNA. Similarly, we confirmed these data using RNase H2-kd nuclear extracts from HeLa cells. As expected, control nuclear extracts (Scramble) displayed a time-dependent endoribonuclease activity on ds_rG:dC, whereas the down regulation of RNase H2 protein expression (siRNase H2) was associated with a marked reduction of the endoribonuclease activity on the same substrate (Figure 7C). On the contrary, once we tested the ability of the nuclear extracts on ds_r8oxoG:dC oligonucleotide, we found only a weak endoribonuclease activity on it (see the band indicated by an asterisk), which was not affected by RNase H2 silencing (Figure 7C). These data support the conclusion that the r8oxoG site in DNA is not recognized by human RNase H2.

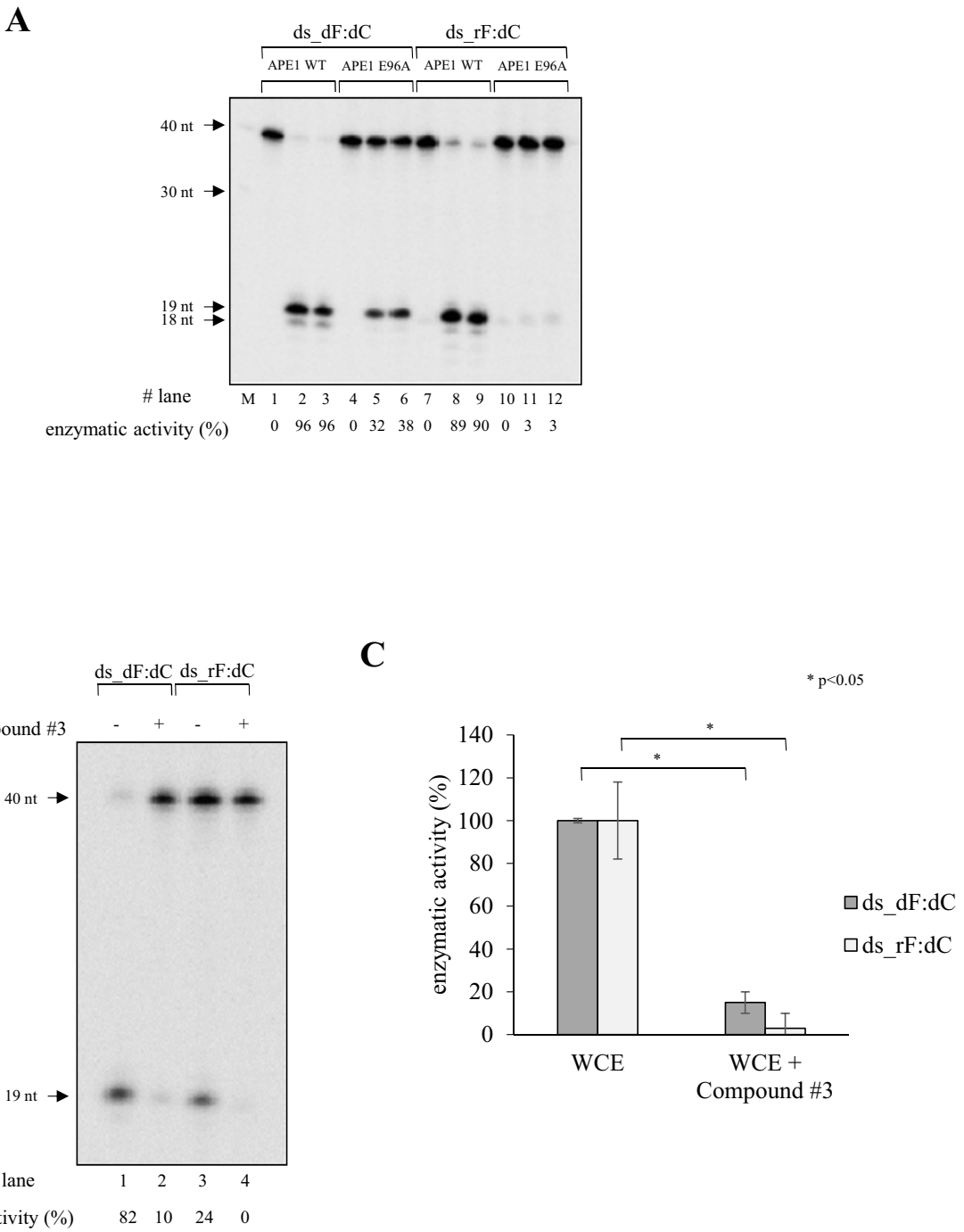

Figure 6. Recombinant human APE1 efficiently processes a tetrahydrofuran ribonucleotide mimicking an abasic residue site (rF) embedded in a duplex DNA substrate. (**A**) Cleavage result of 10 nM of ^{32}P double-stranded oligonucleotides ds_dF:dC or ds_rF:dC substrates with different concentrations of APE1 WT protein (*lanes 1–3* and *7–9*) and mutant APE1 E96A protein (lanes *4–6* and *10–12*) at 37°C for 1 h in APE1-reaction 1× buffer containing 50 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.001 mg/mL BSA and 0.05% Triton X-100. First lane on the left, M is a ssDNA ladder (barely visible) and the black arrows on the left show specific band sizes. Both oligonucleotides were incubated with 0 nM (*lanes 1,4,7,10*), 5 nM (*lanes 2,5,8,11*) or 10 nM (*lanes 3,6,9,12*) of APE1 WT protein (*lanes 1–3* and *7–9*) or mutant E96A protein (*lanes 4–6* and *10–12*), respectively. The percentages of cleavage of each reaction are displayed below the image as enzymatic activity (%). (**B**) Cleavage result of 10 nM ds_dF:dC or ds_rF:dC radioactive substrates using 12.5 ng of whole HeLa cell extracts untreated (*lanes 1, 3*) and treated (*lanes 2, 4*) with 0.20 nM of Compound #3, a specific APE1-endonuclease inhibitor, at 37°C for 10 min. The black arrows on the left of the gel image indicate the size of uncut and cut substrates following denaturation. The cleavage percentage of this experiment is displayed below the image as enzymatic activity (%). (**C**) Histograms showing data from four independent experimental replicas shown in panel (B) with ranges as bars. *P* values of <0.05 are marked by asterisk. WCE, whole cell extracts.

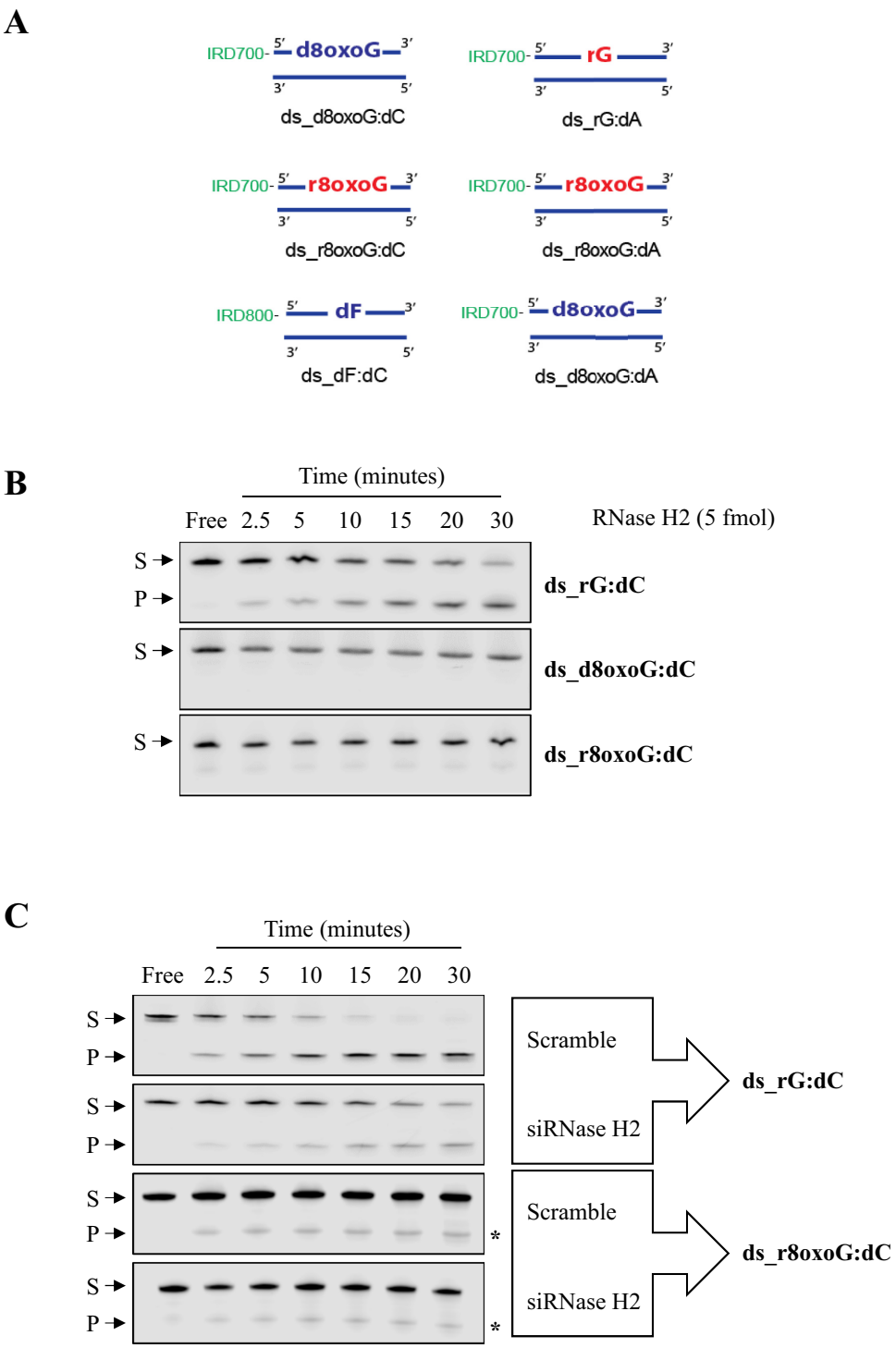

Figure 7. Human RNase H2 is not able to process an r8oxoG site embedded in a duplex DNA substrate. (**A**) Scheme of ds_DNA 25-mer substrates (single strand (ss) oligonucleotide annealed to ss_dC containing- or ss_dA containing-complementary oligonucleotide) containing a d8oxoG, r8oxoG and dF site in the 13th position. The 'IRD700' and 'IRD800' in green indicates IRDye 800 phosphoramidite and IRDye700 phosphoramidite dye tagged at the 5′ end. (**B**) Representative denaturing polyacrylamide gel of oligonucleotides (25 nM) incision by human recombinant RNase H2 (5 fmol). Reaction was performed in RNase H2-buffer (20 mM Tris–HCl, 25 mM KCl, 0.1% BSA, 0.01% Tween20, 4 mM $MgCl_2$, pH 7.4) at 37°C. ds_rG:dC and ds_d8oxoG:dC oligonucleotides were used as positive and negative controls, respectively. Time points are shown on the top of the figure. S indicates the substrate position, while P indicates the product position. (**C**) Representative denaturing polyacrylamide gel of oligonucleotides (0.25 μM) incision by nuclear HeLa cell extracts (NCE). In order to discriminate the activity of RNase H2, 500 ng of NCE in which RNase H2 expression was previously knocked down through specific siRNA (indicated as siRNase H2) were tested in comparison to control cells (Scramble) at different time points (minutes), shown on top of the figure. The endoribonuclease activity detected for r8oxoG oligonucleotide was indicated with an asterisk on the right side of each panel. S indicates the substrate position while P indicates the product position.

OGG1 has neither lyase nor glycosylase activities on oxidized rG substrate

We then tested whether enzymes of the BER pathway may be involved in processing the r8oxoG substrate. To this purpose, we used recombinant purified human OGG1 and APE1 proteins (Supplementary Figure S4A and S5C). OGG1 protein belongs to the bi-functional glycosylases family having both lyase and glycosylase activities on oxidized dG (57). We examined the processing activity of OGG1 on r8oxoG substrate in comparison to the d8oxoG-containing oligonucleotide, as a positive control. First, we tested the ability of OGG1 to recognize an r8oxoG site through electrophoretic mobility shift assay (EMSA). As shown in Figure 8A, increasing amount of recombinant OGG1 formed a stable retarded complex with the r8oxoG oligonucleotide in a dose-dependent manner. As confirmed in the data shown in Supplementary Figure S5E, OGG1 binding was specific for the modified r8oxoG- containing oligonucleotide (*lanes 2 and 3*). Indeed, the same retarded band was observed when the recombinant OGG1 was incubated with the positive control ds_d8oxoG:dC (*lanes 11 and 12*) but not with the negative controls: ds_rG:dC (*lanes 5 and 6*) and ds_dG:dC (*lanes 8 and 9*).

Furthermore, we tested the lyase activity of OGG1 on different substrates. Figure 8B shows that when we incubated increasing amounts of recombinant protein for 30 min at 37°C with different substrates, OGG1 was able to process only the canonical substrate ds_d8oxoG:dC in a dose response manner (Supplementary Figure S5D), whereas no lyase activity was apparent for any of the other substrates used, including the ds_r8oxoG:dC and the ds_r8oxoG:dA.

Since OGG1 is the major glycosylase enzyme in the BER pathway, coordinating with the downstream endonuclease APE1, which is able to recognize and process the abasic site generated by the glycosylase activity of OGG1 (28,58,59), we measured its glycosylase activity on the same substrates using recombinant purified APE1 (Figure 8C). In this case, we co-incubated a fixed amount of recombinant OGG1 with increasing amounts of APE1 for 30 min. While OGG1 displayed a robust glycosylase activity on the canonical ds_d8oxoG:dC substrate, particularly in the presence of the APE1 protein, we detected only a weak activity on ds_r8oxoG:dC oligonucleotide (indicated by a single asterisk) (Figure 8D). Moreover, the presence of an additional higher mobility band, increasing as a function of APE1 concentration (indicated with a double asterisk) was observed only in the case of the ds_r8oxoG:dC substrate. We conclude that OGG1 has neither lyase nor glycosylase activity on the r8oxoG substrate, and that APE1 can weakly process this substrate alone. About APE1 activity, a detailed description is explained in the next paragraph.

APE1 has a weak endo-/exo-nuclease activities on the r8oxoG-containing substrate depending on Mg^{2+} concentration and on the presence of its N-terminal domain

Based on the above presented data, we then checked whether APE1 '*per se*' had any endoribonuclease activity on ds_r8oxoG:dC substrate (Figures 9 and 10). Compared to the ds_dF:dC substrate, APE1 displayed a modest, though significant, processing activity on both ds_r8oxoG:dC and ds_r8oxoG:dA oligonucleotides, while no activity was observed in the case of the dG- and the d8oxoG-containing substrates (Figure 9, panels A and B), as expected. As observed above, the appearance of an additional faster migrating cleavage product (indicated by a double asterisk corresponding to a 11-nt product in Figure 8D) was visible in the case of the ds_r8oxoG:dC substrate, which might be associated with a recently identified 3′-exonuclease activity by the protein (42). We checked the occurrence of the cleavage at the expected ribonucleotide sites, by oligonucleotide sequences of increasing length, ranging from 10 to 16 nucleotides, as molecular markers (Supplementary Figure S6A) and through alkaline hydrolysis experiments (Supplementary Figure S6B). As it is visible (Supplementary Figure S6A), cleavage products of the ds_r8oxoG:dC oligonucleotide were of the expected size and comprised between 11- and 12-nucleotides and are thus compatible with endonucleolytic cleavage occurring only at the 5′ side of the lesion (fragment 12-nt long) and with a 3′-exonuclease activity giving rise to the fragment of 11-nt long. In order to exclude that the observed cleavage product was due to the processing of a residual non-annealed oligonucleotide possibly present after the annealing reaction, we incubated APE1 protein with single stranded oligonucleotide (ss_r8oxoG) and compared the cleavage product with the annealed oligonucleotide (ds_r8oxoG:dC). Comparing the result with both ss_dF and ds_dF:dC oligonucleotides, a product was detectable only using the double stranded oligonucleotides as substrates. No bands were observed using the ss_r8oxoG oligonucleotide (Supplementary Figure S6C) demonstrating the requirement for secondary structured oligonucleotide sequences for efficient enzymatic activities by APE1. In contrast, using the oligonucleotide containing the mismatched ds_r8oxoG:dA, most of the fragments produced after incision by the AP endonucleolytic activity (indicated with a single asterisk) were not further degraded by the exonucleolytic activity (Figure 9A). Therefore, these data demonstrate that the APE1 enzymatic activity on the r8oxoG substrate requires a dsDNA molecule and exonuclease activity is dependent on the paired nucleotide, possibly as a consequence of a different stereochemical geometry between the 8oxoG:A and the 8oxoG:C.

It has been previously demonstrated that the exonuclease activity of APE1 strictly depends on salt concentrations (60). We therefore tested whether the 3′-exonuclease activity observed on the ds_r8oxodG:dC shared some common features (in terms of dependence on the ionic strength conditions) with the 3′-exonuclease activity on mispaired DNA, as previously described (60). Firstly, we determined the optimal $MgCl_2$ (Figure 10A) and KCl (Figure 10B) concentrations required for the 3′-exonuclease activity. Indeed, the 3′-exonuclease activity was present up to a concentration of 2 mM $MgCl_2$. An inhibitory effect was apparent at $MgCl_2$ concentrations above 4 mM. At the same time, the 3′-exonuclease activity was poorly affected at KCl concentration equal to 100 mM. These results are in line with previous data on 3′-mispaired DNA (60) and suggest that the observed 3′-exonuclease activity strongly depends on the electrostatic interaction of APE1 with the substrate during the cleavage reaction and with the role of Mg^{2+} ions.

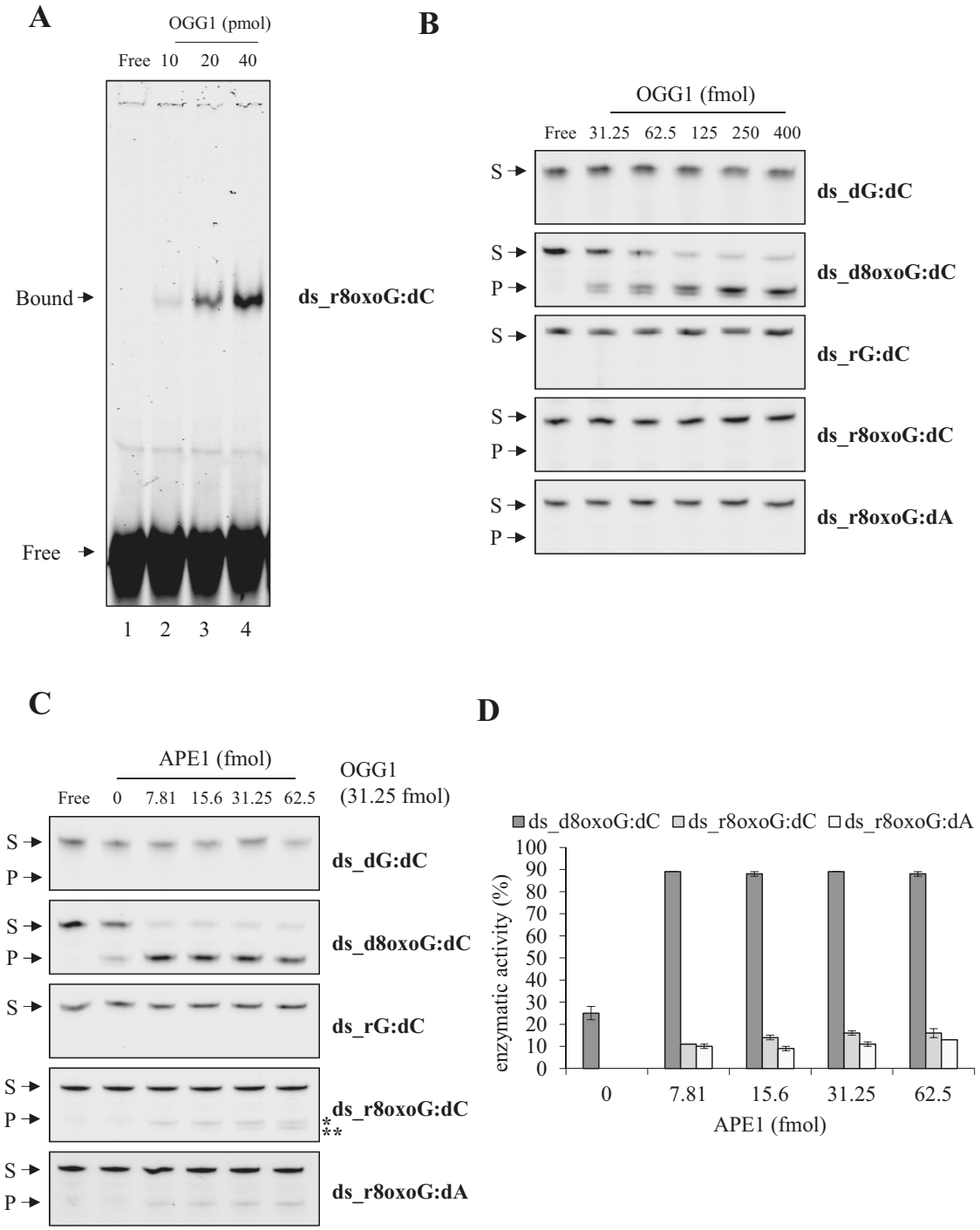

Figure 8. Human OGG1 has neither lyase nor glycosylase activities on r8oxoG-containing oligonucleotide. (**A**) Representative native EMSA polyacrylamide gel of OGG1 binding on ds_r8oxoG:dC oligonucleotide (25 nM) is shown. The 'Bound' arrow indicates the retarded complex between OGG1 and the probe whereas the 'Free' arrow the unbound substrate. Amounts of OGG1 protein, expressed in *pico* moles, are shown on the top of the figure. Reactions were performed as explained in 'Materials and Methods' section**.** (**B**) Representative denaturing polyacrylamide gel of lyase activity of human OGG1 on different duplex DNA oligonucleotides (25 nM). Doses of OGG1 protein expressed in *femto* moles are shown on the top of the figure. Reactions were performed in OGG1-buffer (20 mM Tris–HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4) at 37°C for 30 min. S indicates the substrate position, while P indicates the product position. (**C**) Representative denaturing polyacrylamide gel of incision by different doses of APE1 co-incubated with a fixed amount of OGG1 (3.125 nM) on different duplex DNA oligonucleotides (25 nM) in order to investigate glycosylase activity of OGG1. Different doses of APE1 protein, expressed in *femto* moles, are shown on top of the figure. Reactions were performed in a buffer containing 20 mM Tris–HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4 at 37°C for 30 min. S indicates the substrate position while P indicates the product position. Moreover, at the right of the panel, a longer product of about 12 nucleotides is indicated by an asterisk whereas a smaller one of 11 nucleotides is indicated by a double asterisk. (**D**) Histograms represent the dose response of OGG1 glycosylase activity on ds_d8oxoG:dC and paired and mismatched ds_r8oxoG oligonucleotides. ds_d8oxoG oligonucleotide was used as a positive control whereas ds_dG:dC and ds_rG:dC oligonucleotides were used as negative controls. The activity is reported as percentage of substrate converted to product. Data are expressed as mean ± SD of three independent technical replicas.

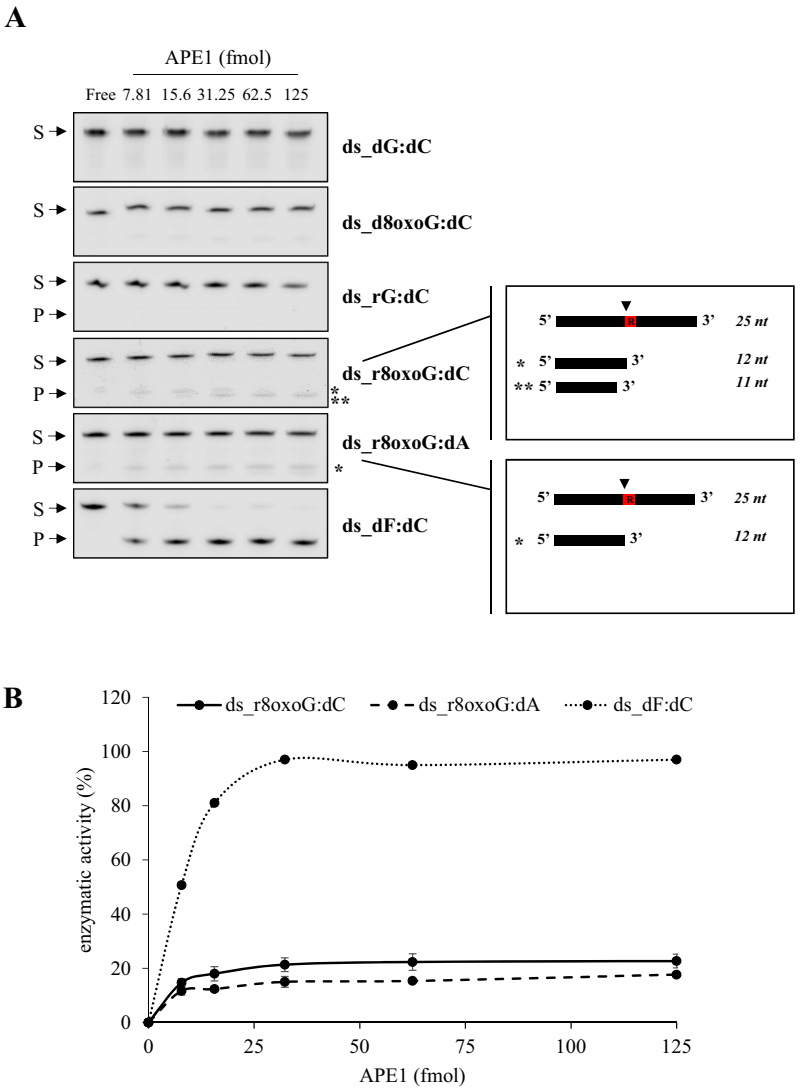

Figure 9. Human APE1 shows a weak endo- and a 3′-exonuclease activities on r8oxoG substrate. (**A**) Representative denaturing polyacrylamide gel of APE1 incision on different duplex DNA oligonucleotides (25 nM), in which ds_dF:dC oligonucleotide was used as a positive control, whereas ds_dG:dC and ds_rG:dC oligonucleotides were used as negative controls. The doses of APE1 protein used, expressed in *femto* moles, are shown on the top of the figure. On the right side, a schematic representation of the cleavage products, showing the position of the ribonucleotide (red box with R) embedded in the DNA oligonucleotide and the APE1 cleavage on it, producing a longer product of about 12 nucleotides (*) and a smaller one of 11 nucleotides (**). Reactions were performed in APE1-buffer containing 20 mM Tris–HCl, 100 mM KCl, 0.1% BSA, 0.01% Tween20, pH 7.4 for 30 min at 37°C. S indicates the substrate position while P indicates the product position. (**B**) Relative graph indicates a dose–response APE1 activity on paired and mismatched ds_r8oxoG:dC oligonucleotide in comparison to ds_dF:dC positive control. Data are expressed as mean ± SD of three independent technical replicas.

After choosing the optimal salts conditions, in which both endo- and exo- activities of APE1 (100 mM KCl and 1 mM $MgCl_2$) are present, we then evaluated whether the enzymatic activity of APE1 on ds_r8oxoG:dC was dependent on the same catalytic site responsible for the endonuclease activity observed on abasic dsDNA and abasic rNMP. To this aim, the enzymatic activity of the E96A mutant was compared to that of the WT protein (Supplementary Figure S6D). These data demonstrate that the APE1 E96A mutant has a reduced endoribonuclease activity showing no 3′-exonuclease activity over the ds_r8oxoG:dC substrate. Due to the effect of salt concentration on this latter activity, we also tested the enzymatic activity of the APE1 NΔ33 deletion mutant (Supplementary Figure S6D). Interestingly, while this protein retained the endoribonuclease activity of the WT protein, its 3′-exonuclease activity was abolished. Moreover, treatment with APE1 inhibitor Compound #3 confirmed that the catalytic site responsible for the endonuclease activity is also responsible for the endoribonuclease activity over the r8oxoG substrate (Supplementary Figure S6E). These results show that APE1 has a weak, though significant, endoribonuclease activity on r8oxoG substrates with an additional specific 3′-exonuclease activity dependent on: (i) the kind of pair: i.e. ds_r8oxoG:dC or ds_r8oxoG:dA; (ii) salt concentrations (i.e. Mg^{2+}); (iii) the presence of the 33 N-terminal domain.

Overall, our data demonstrate that BER enzymes but not RER are involved in the processing of non-canonical rN-

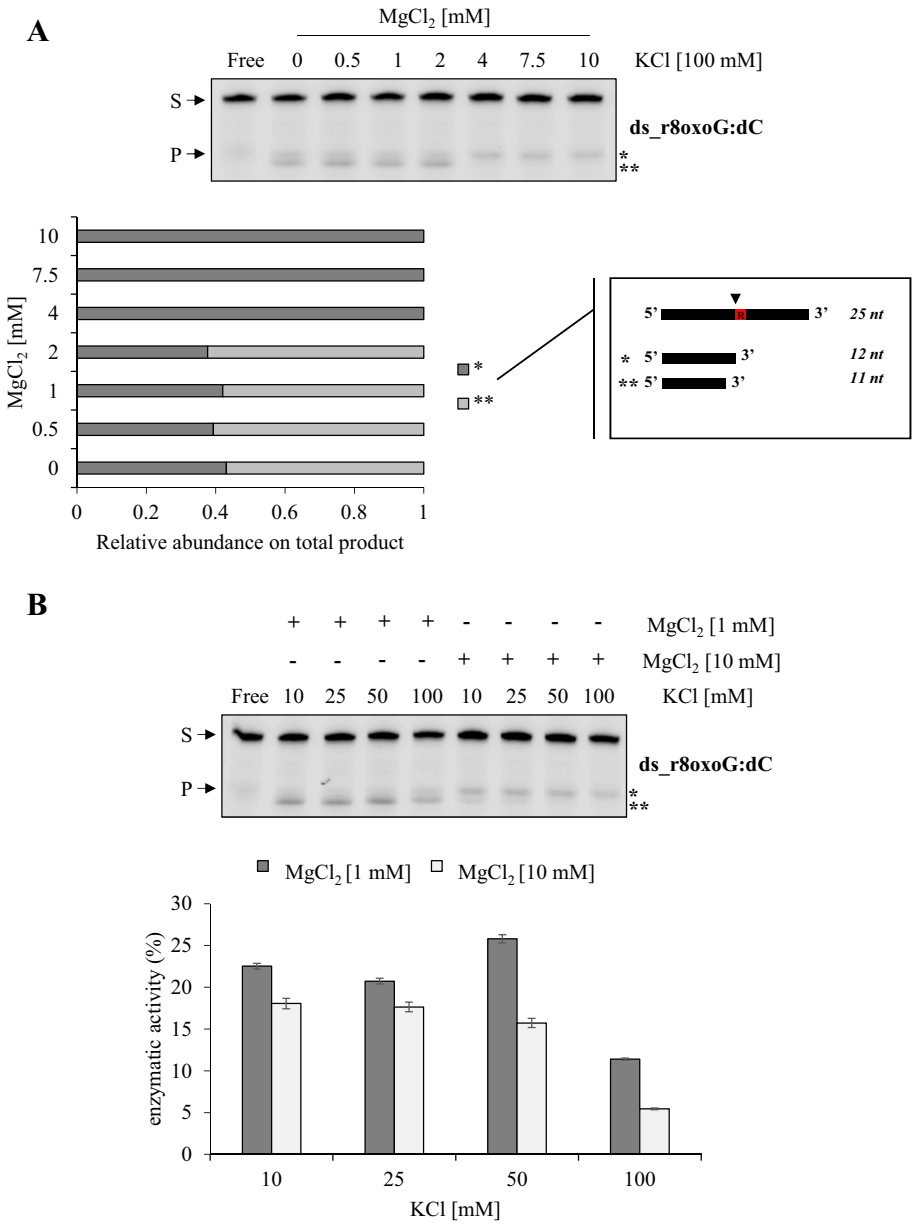

Figure 10. Human APE1 activities on r8oxoG substrate depend on mono- and di-valent cations. (**A**) Representative denaturing polyacrylamide gel of APE1 (5 nM) incision on ds_r8oxoG:dC oligonucleotide under different $MgCl_2$ concentrations that is expressed in mM (*top*) performing a reaction long 30 minutes in APE1 buffer. S indicates the substrate position while P indicates the product position. Moreover, at the right of the panel, a longer product of about 12 nucleotides is indicated by an asterisk whereas a smaller one of 11 nucleotides is indicated by a double asterisk Also shown on the right is a schematic representation of the cleavage products, showing the position of the ribonucleotide (red box with R) embedded in the DNA oligonucleotide and the APE1 cleavage on it, producing a longer product of about 12 nucleotides (*) and a smaller one of 11 nucleotides (**). Relative graph shows the ratio between two products obtained as a function of $MgCl_2$ concentration (*bottom*). (**B**) Representative denaturing polyacrylamide gel of APE1 (5 nM) incision on ds_r8oxoG:dC oligonucleotide under different KCl concentrations in combination with two different $MgCl_2$ concentrations, 1 mM and 10 mM (*top*) performing a reaction long 30 min in a buffer containing 20 mM Tris–HCl, 0.1% BSA, 0.01% Tween20, pH 7.4. S indicates the substrate position, while P indicates the product position. Moreover, at the right of the panel, a longer product of about 12 nucleotides is indicated by an asterisk whereas a smaller one of 11 nucleotides is indicated by a double asterisk. Relative graph shows product levels in association with different salts concentrations (*bottom*).

MPs, such as abasic or oxidized, incorporated in DNA. Because the repair pathway catalyzed by APE1 toward the oxidized rG is fundamentally different from that of BER, since no bases are excised, we can conclude that the observed effect could be ascribed to an alternative damage-specific endonuclease initiated repair pathway, previously referred to as either alternative excision repair (AER) or nucleotide incision repair (NIR) (reviewed in (61,62)).

APE1 and RNase H2 do not biochemically and functionally interact in human cells

To support the functional independence between BER and RER, we tested whether APE1 and RNase H2 proteins

functionally interact. We tested whether this hypothesis was confirmed in HeLa cells transfected with siRNAs specific for APE1 and RNase H2 mRNAs to knock down the corresponding endogenous proteins. Whole cell extracts were prepared as explained in Materials and Methods section, and western blotting analysis was used to check the effective protein down regulation (Supplementary Figure S7A). Then, we checked the ability of APE1 and RNase H2 from cell extracts to recognize the ds_r8oxoG:dC substrate. We performed EMSA with cell extracts from control (Scramble) and APE1-kd (siAPE1) or RNase H2-kd (siRNase H2) cells. As demonstrated in Supplementary Figure S7B, incubation of cell extract from control cells displayed a retarded band containing APE1-complex, as also demonstrated by supershift EMSA experiments with anti-APE1 specific antibody (Supplementary Figure S7, panels C and D). The intensity of the retarded complex was decreased upon APE1-kd (siAPE1) (Supplementary Figure S7B, lane 4) and upon the double APE1/RNase H2-kd (siAPE1 + siRNase H2) (*lane 6*) but not upon RNase H2 silencing alone (siRNase H2) (*lane 5*), confirming that APE1 is involved in a protein complex able to recognize r8oxoG damage in which RNase H2 is not present.

DISCUSSION

Increased body of evidence supports the notion that incorporation of rNMPs in DNA is a frequent phenomenon, having profound detrimental effects on genome stability of both prokaryotes and eukaryotes (1,2,63,64). In humans, as well as in yeast and bacteria including Archaea, the main processing pathway responsible for repairing of these lesions is the RER pathway, which involves the RNase H2 enzyme (65,66). RNase H2 importance in higher organisms is testified by its essentiality for embryonic development in mouse (67). Moreover, RNase H2 mutations in humans are causally linked to the onset of AGS, a rare autoimmune inflammatory disease (68,69). It can be hypothesized that among the many millions rNMPs that are introduced in the mammalian genome per cell cycle (3), not only canonical rNMPs are incorporated but also damaged rNMPs (such as abasic and oxidized). Indeed, like deoxyribonucleotides, rNMPs are also susceptible to oxidative insults (5,70), and a significant generation of abasic sites formation has been demonstrated upon RNA oxidation and alkylation (71). While the role of RNase H2-initiated RER mechanism of DNA repair in recognizing and cleaving rNMPs embedded in DNA is well established (3,15), nothing is known regarding the DNA repair pathways involved in the removal of damaged rNMPs.

BER is the main mechanism coping with the repair of non-distorting single-base lesions, such as abasic sites and oxidized bases (27). Interestingly, emerging literature, including ours, pointed to a new function of BER in RNA quality control surveillance and RNA-decay with the functions of SMUG1, PARP1 and APE1 in RNA processing (38,72). At present, however, there is no evidence that BER may cope with the removal of rNMPs from DNA. Identifying whether BER may target unmodified and/or modified rNMPs in DNA is important to better understanding the mechanism of genotoxicity of oxidative stress and the impact of BER defects in human disease, cancer mechanisms, and for the development of new anticancer strategies.

In this work, we demonstrate that an rAP site embedded in DNA is targeted by APE1 of BER rather than RER in eukaryotic systems (Figure 11A). We have found that eukaryotic RNase H2 enzymes from yeast, mouse and human, and from human cell extracts are unable to process rAP sites in DNA, whereas recombinant human APE1 is able to efficiently cleave this type of damage (Figures 2 and 3). APE1 processes the rAP site as efficiently as the canonical deoxy-abasic site as measured by kinetic data (Table 1). Data using catalytic inactive APE1 mutants (E96A) clearly demonstrate that the endonuclease active site of APE1 is required to perform the endoribonuclease activity on a ribose abasic site in dsDNA. Differently, the cleavage activity of the 33N-terminal truncated mutant, which does not impact the catalytic function of the enzyme but is involved in the release of the product upon cleavage (48), is comparable to that of wild-type APE1. This last result demonstrates that the unstructured N-domain is dispensable for the enzymatic reaction on the abasic ribonucleotide site. Moreover, using HeLa nuclear cell extracts that were siRNA-depleted of APE1 or RNAse H2 proteins, we showed that the processing activity of the rAP site in DNA depends only on the presence of APE1 and not on RNase H2. These results highlight a new role of APE1 in repairing rAP sites embedded in DNA, demonstrating that the catalytic site of APE1 and the mechanism of product release is similar to that of the canonical deoxy-substrate.

rAP sites embedded in DNA may be generated by spontaneous hydrolysis or by the action of an unknown glycosylase on oxidized rNMPs, such as r8oxoG. Furthermore, a potentially significant, yet poorly characterized, source of rNMPs incorporated in DNA is the oxidative stress. rNMPs were shown to form during oxidative DNA damage both *in vitro* and *in vivo* (5). Therefore, it is also possible that abasic and oxidized DNA is converted into RNA. It was estimated that spontaneous depurination occurs 1,000 times slower in RNA than DNA (73). For example the rate of depurination in DNA under physiological conditions is estimated to be 1 out of 100 000 purines every cell cycle. This rate gives 10 000 abasic sites per day in human cells (74). Considering the remarkable abundance of rNMPs in DNA, which could be as many as 600 000 rNMPs in budding yeast genomic DNA, and therefore a factor of 250 higher in mammalian genomic DNA (150 000 000) (3), i.e. >300 millions in the human diploid DNA per cell cycle, it is not unrealistic to anticipate that cells may contain a non-negligible number of rAP sites in DNA, or oxidized rNMPs. Interestingly, abasic RNA results significantly more stable than abasic DNA, suggesting that specific enzymatic mechanisms should exist *in vivo* to cope with this harmful lesion (75). In addition, in conditions of oxidative DNA damage, such as in cancer cells, the likelihood of such base modifications can increase. Moreover, recent studies already suggested that r8-oxoGTP is formed *in vivo* under oxidative stress conditions and may be incorporated during replication into DNA by *S. pombe*, *M. smegmatis* and human (34–36). Moreover, the introduction of r8oxoG in DNA can be catalyzed by human DNA Polβ (36). However, the level and function of Polβ in cells are highly regulated by complex signaling mecha-

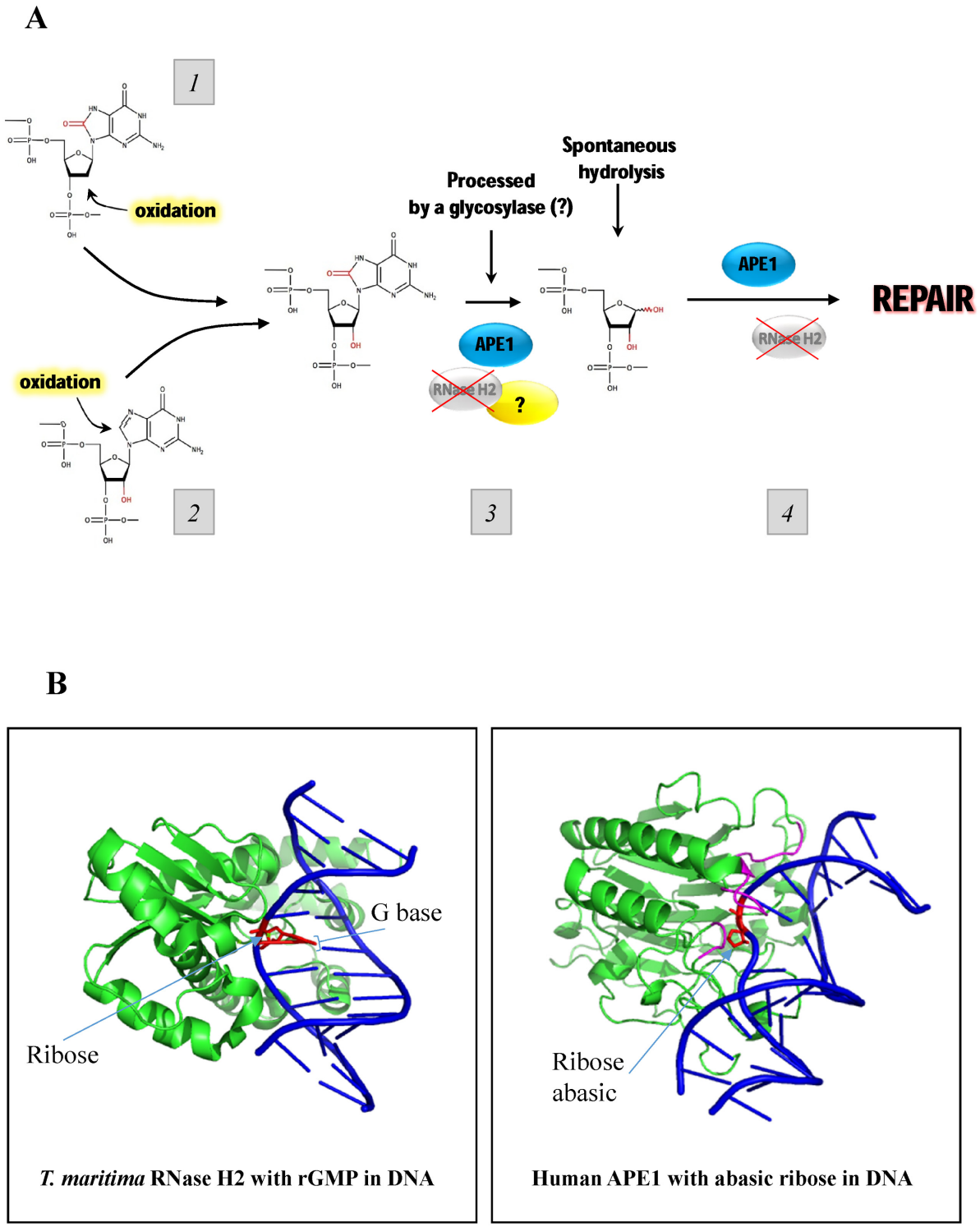

Figure 11. Model for repair of oxidized rNMPs and rAP sites embedded in DNA by APE1. (**A**) 8oxo-ribonucleotides could be generated in the cellular nucleotide pool or even when they are already incorporated in DNA as a result of an oxidation of the sugar (1) and/or the base (2). After the evidence that human RNase H2 is not able to process an oxidized rNMP embedded in DNA, we found that APE1 shows a weak but significant activity on it (3). Similarly, RNase H2 does not process an rAP embedded in DNA, which could be generated spontaneously or by the r8oxoG processing, and again APE1 possesses a strong activity on this type of damage (4). Because APE1 activity on 8oxo-ribonucleotides in DNA is low, we hypothesize that other proteins (some glycosylases?) may participate in their repair. (**B**) Structural models with the active site of RNase H2 and APE1 with an rNMP or an rAP. *T. maritima* (left) and human APE1 (right) is in a complex with DNA having a single rNMP or single abasic residue, respectively. DNA is indicated in blue, while the single rNMP and the abasic residues are shown in red as sticks. The arrows points towards ribose or G base or abasic sites in the panels. Proteins are shown in green except for three of the several regions on APE1 that engulf the abasic sugar which are in magenta. PDB for RNase H2 is 303-F; for APE1 as 1DEW (92).

nism and interactome networks (76). Therefore, the probability of r8oxoG incorporation into genomic DNA by Polβ is still a question under debate, which needs further experimental proof. Spontaneous formation of rAP sites being a rare event, it is a key question to find activities producing rAPs following processing of oxidized rNMPs in DNA. In addition, a possible RER and BER involvement in removal of r8oxoG from DNA has been proposed in other reports (36,77). Work in our laboratory is ongoing along these lines to address these fundamental issues. Considering these observations, we focused our attention on which BER protein, if any, may be involved in recognition and cleavage of oxidized rNMPs (r8oxoG) embedded in DNA. First, we tested the RNase H2 activity on r8oxoG substrate (Figure 7). Similarly to results with the abasic rNMP in DNA our data clearly demonstrate that RER is not involved in processing of oxidized rNMPs embedded in DNA. Based on these findings, we explored a potential role of the BER pathway. First, we showed that the human OGG1, the main glycosylase enzyme able to recognize and repair oxidized dG through its lyase and glycosylase activities, has neither a lyase nor a glycosylase activity on an oxidized rG site embedded in a DNA substrate, despite its ability to efficiently bind the oxidized substrate (Figure 8), in agreement with recent findings (77). Interestingly, we discovered that APE1 has a weak endoribonuclease activity on r8oxoG site embedded in a DNA substrate, and shows a 3′-exonuclease activity (Figures 9 and 10), similarly to the 3′-exonuclease activity on DNA demonstrated previously (52,54). In line with previous results, the 3′-exonuclease activity of APE1 is strictly dependent on Mg^{2+} concentration and on the presence of the first 33 aminoacids. The importance of the N-terminal domain is to be attributed to different reasons: (i) it bears the majority of the positive charges of APE1; (ii) is the target of the main post-translational modifications of the protein (i.e. acetylation, ubiquitination, proteolysis); (iii) is involved in modulating the interaction with different protein partners and finally (iv) may modulate the catalytic rate, probably acting on the k_{off} of the catalytic reaction due to increased speed of product release (37,48,78). These unexpected results, which suggest that APE1 3′-exonuclease activity strongly depends on the electrostatic interaction of APE1, involving its unstructured N-terminal domain, with the substrate (52), may be explained on the basis of the previously characterized ability of APE1 to process some particular structured RNA species in a specific manner (37).The activity of APE1 on r8oxoG embedded in DNA does not hide the possibility that a similar activity could be exerted on RNA molecules too. This finding could represent the first demonstration of an enzyme able to recognize and process oxidized RNA (78). To date, RNA oxidation has been shown to exert detrimental physiological effects and to be a common feature in different human pathologies ranging from ageing to neurodegenerative and cancer diseases (79). For instance, oxidized RNA (80) or RNA containing abasic sites (75) show inhibitory effects on reverse transcriptase activity, whereas oxidized mRNA (81,82) or mRNA with abasic sites (83) exhibit compromised translation activity as well as translation fidelity (84). The weak endo- and 3′-exonuclease activities on r8oxoG- containing substrate and their dependence on Mg^{2+}-concentrations and on the presence of the first N-terminal domain residues of APE1 are fully in agreement with the previously described nucleotide incision repair (NIR) function by APE1 on several oxidized substrates, such as: 5,6-dihydro-2′-deoxyuridine, 5,6-dihydrothymidine, 5-hydroxy-2'-deoxyuridine, 5-hydroxycytosine (43–45). Notably, the limited activity obtained in the experimental conditions we used (also after changing the pH conditions, data not shown) are in agreement with previous reports on the NIR function by APE1 on some particular substrates such as the αdG:dG and the 5OH-dC:dG (44). In addition, the biochemical characterization through MALDI-MS and HPLC analyses we performed (Supplementary Figure S5 panels A and B) may be suggestive for the existence of an equilibrium between different conformational species of r8oxoG dsDNA, excluding any possible bias due to contaminant present in the oligonucleotide used for the assays. Therefore, these findings underscore the importance of identifying the enzyme(s) responsible for the recognition and efficient processing of the r8oxoG substrate, in order to further extend our studies and our understanding of this hot scientific topic.

We observed that OGG1 is unable to process the r8oxoG substrate, while being perfectly able to specifically bind it, similar to what recently published by Sassa *et al*. (77). At present, it is possible to speculate that though the base-flipping occurs, the enzymes is unable to hydrolyze the N-glycosidic bond and has no lyase activity maybe as a consequence of the steric hindrance with the 2′OH of the ribose which renders the C1′ unavailable for the nucleophilic attack by the catalytic site. Differently from our results, Sassa *et al*. found no enzymatic activity by APE1. This discrepancy with our results may be due to the different experimental conditions for the enzymatic assays, i.e. higher Mg^{2+} concentrations and the use of a small amount of EDTA, both aspects already demonstrated to strongly affect the APE1 enzymatic activity on the r8oxoG substrate. A comparative experiment we performed (Supplementary Figure S8) was indeed supportive of this hypothesis, reinforcing the importance of the experimental conditions when studying the non-canonical functions of APE1 protein. Interestingly, Sassa *et al.* showed that the commercially available prokaryotic RNase HII preserves the ability to remove an oxidized rNMP in a DNA duplex. Contrary to these results, our findings show that eukaryotic RNase H2 is completely inactive on a substrate containing an oxidized rNMP. These data suggest that the ability to process r8oxoG in DNA has been lost during evolution and deserves further studies.

To explain why APE1 recognized the abasic rNMP in DNA and RNase H2 did not, we compared the model structures of DNA with an rNMP or an rAP site in the RNase H2 and APE1 active site, respectively (Figure 11B). RNase H2 recognizes the RNA-DNA junction with the substrate participating in catalysis. Prior to incision by RNase H2, the rNMP base is hydrogen bonded to the complementary DNA strand base (85). If the rNMP is abasic, there is no hydrogen bonding to stabilize the complex required for RNase H2. Rather, an orphan base on the complementary DNA strand is present. We hypothesized that the lack of the hydrogen bonding between the abasic rNMP and the opposite deoxyribonucleotide interferes with the capacity of RNase

H2 to recognize an abasic rNMP and cleave it. Thus, the role of recognizing and cleaving abasic rNMPs is not specific of RNase H2. Differently from RNase H2, APE1 specifically recognizes and cleaves an abasic distortion in DNA, and basically engulfs the sugar-phosphate further distorting the DNA (86), as seen in Figure 11B. Here, we predicted that the ribose extra OH would have only minor influence on the structure. Therefore, this could explain why the abasic rNMP, like an abasic deoxyribonucleotide in DNA, was efficiently cleaved by APE1.

Experiments are underway in order to address if other glycosylases may process oxidized rNMPs in order to generate rAP sites, which are then efficiently processed by APE1. We previously demonstrated that APE1-defective cells have increased oxidized rRNA content upon oxidative stress (37). This result has now a molecular explanation in the observed endoribonuclease activity of APE1 over r8oxoG containing oligonucleotides and will deserve further attention in our future studies. Regarding the formation of abasic ribonucleotides in DNA, this is still a matter of debate. The existence of specific N-ribohydrolases, including the toxin ricin, has been already documented (87) to be able to generate abasic rNMPs in RNA molecules, besides spontaneous generation (71). A role for the YB-1 protein in recognizing oxidized ribonucleotides sites in RNA has also been hypothesized (88), but no specific enzymatic mechanisms able to remove the oxidized base has been described, yet. The accumulation of the r8oxoG substrates, which occurs on RNA upon silencing of APE1 expression, may thus be explained under the assumption that enzymatic removal of oxidized rNMPs may represent the limiting step in the process. Besides its direct activity on r8oxoG, APE1 could be stimulated by a glycosylase activity allowing a faster turnover as demonstrated for DNA substrates (89). Work is in progress along these lines to better inspect this mechanism and the putative glycosylase enzymes involved. Moreover, since APE1 is overexpressed in different types of cancer, such as ovarian, gastro-esophageal, pancreatico-biliary, lung and breast cancers (90,91), it would be interesting to determine whether any correlation exists between its expression level and presence of modified rNMPs in cancers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Unveiling the non-repair face of the Base Excision Repair pathway in RNA processing: A missing link between DNA repair and gene expression?

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ABSTRACT

The Base Excision Repair (BER) pathway, initially studied as a mere DNA repair pathway, has been later found to be implicated in the expression of cancer related genes in human. For several years, this intricate involvement in apparently different processes represented a mystery, which we now are starting to unveil.

The BER handles simple alkylation and oxidative lesions arising from both endogenous and exogenous sources, including cancer therapy agents. Surprisingly, BER pathway involvement in transcriptional regulation, immunoglobulin variability and switch recombination, RNA metabolism and nucleolar function is astonishingly consolidating. An emerging evidence in tumor biology is that RNA processing pathways participate in DNA Damage Response (DDR) and that defects in these regulatory connections are associated with genomic instability of cancers. In fact, many BER proteins are associated with those involved in RNA metabolism, ncRNA processing and transcriptional regulation, including within the nucleolus, proving a substantial role of the interactome network in determining their non-canonical functions in tumor cells. Maybe these new insights of BER enzymes, along with their emerging function in RNA-decay, may explain BER essential role in tumor development and chemoresistance and may explain the long-time mystery. Here, we would like to summarize different roles of BER pathway in human cells. First, we will give a short description of the classical BER pathway, which has been covered in detail in recent reviews. We will then outline potential new roles of BER in gene expression and RNA metabolism. Although recent works have provided tremendous amount of data in this field, there are still lot of open questions.

1. Relevance of the canonical BER pathway and open questions

The BER pathway (Fig. 1) is an essential DNA repair system in higher eukaryotes and gene deletions of the core BER factors (apurinic/apyrimidinic endonuclease 1 – APE1, DNA polymerase β − Polβ, X-ray repair cross-complementing 1 – XRCC1, DNA ligase I − LigI and DNA ligase III − LigIII) results in embryonic or early post-natal lethality [1]. The pathway is comprised of five major steps, in which enzymatic and non-enzymatic components cooperate to carry out a highly integrated set of reactions: i) recognition and excision of the damaged base; ii) incision of the resulting AP site to generate a nick on the DNA backbone; iii) processing of the nick ends; iv) filling of the nucleotide gap; and v) sealing of the nick (Fig. 2).

Different specific DNA glycosylases scan the DNA substrate, recognize, through a flipping out mechanism, and excise the damaged base in a lesion-specific manner. Two kinds of DNA glycosylases are known: mono- or bi-functional, depending on their mechanism of action. While monofunctional DNA glycosylases (e.g. the uracil-DNA glycosylase − UNG) simply cleave the C1′-N-glycosidic bond, generating an AP-site, bifunctional enzymes also possess an associated β-lyase activity (e.g. the 8-oxoguanine DNA glycosylase − OGG1) deputed to cleave the DNA backbone leaving a 3′-α,β-unsaturated aldehyde blocking group. An additional family of DNA glycosylases, represented by the human NEIL1 and NEIL2 enzymes, is also able to operate a β,δ-elimination reaction, leaving a 3′-phosphate nick [2]. Higher eukaryotes are provided with a vast array of DNA glycosylases with a significant redundancy in their damage selectivity for this reason, single knockout of several DNA glycosylases is not lethal *per se*, although an accumulation of unrepaired DNA lesions occurs [3].

The glycosylase-catalyzed reaction generally produces an AP-site, which is immediately processed by APE1 in metazoans. APE1 cleaves at the 5′ termini of the abasic site, generating a nick on the DNA backbone

Abbreviations: AP-site, apurinic/apyrimidinic site; APE1, apurinic/apyrimidinic endonuclease 1; BER, Base Excision Repair; DDR, DNA damage response; miRNA, microRNA; ncRNA, non coding RNA; NIR, Nucleotide Incision Repair; PARP, poly(ADP-ribose) polymerase; RBP, RNA binding protein; rNMPs, ribonucleotides; ROS, reactive oxygen species; SSBR, Single Strand Break Repair

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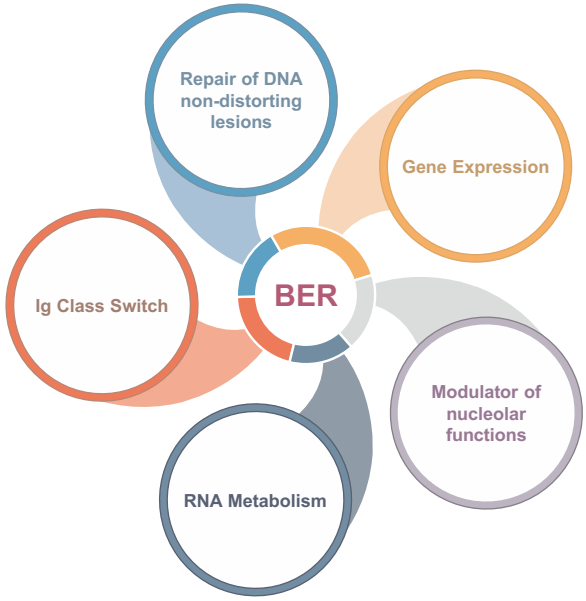

Fig. 1. BER exerts different functions in human cells.
Schematic representation of different biological functions of Base Excision Repair (BER) pathway in human cells including DNA repair player of alkylated and oxidative DNA lesions; regulator of expression of genes involved in response to genotoxicants; regulator in RNA metabolism, control of nucleolar function and regulation of immunoglobulin Class Switching together with AID.

and producing a 3′-OH and a 5′-dRP (deoxyribonucleotide-phosphate) termini. Usually, APE1-incision activity is sufficient to generate the DNA ends required for the completion of the DNA repair process. Once further oxidation of the DNA termini or base-excision operated by bifunctional glycosylases occurs, other end-processing enzymes may be involved, such as tyrosyl-DNA phosphodiesterase 1 (TDP1), aprataxin (APTX) or polynucleotide kinase 3′-phosphatase (PNKP). The dRP-lyase activity of Polβ, along with APE1 3′-phosphodiesterase activity, contributes to the "end-cleaning" process, ultimately generating a single-nucleotide gap that can be efficiently filled in and re-ligated [4].

BER is then completed via a "short-patch" (SP) or a "long-patch" (LP) pathways, depending on the 5′-moiety generated. In the SP-BER, Polβ is engaged to replace the missing nucleotide, and then is followed by the XRCC1-LigIII complex, which is responsible for the ligation of the nick [5]. In the presence of a 5′-moiety refractory to the Polβ lyase activity, low ligation efficiency, or during the S-phase of the cell cycle (i.e. when replication-associated proteins are more abundant), BER can be completed through the LP-BER which involves a strand displacement-dependent gap filling process [1]. Replicating polymerases, such as DNA polymerase δ and ε, act in concert with the sliding clamp PCNA (proliferating cell nuclear antigen) in the LP-BER, generating a stretch of 2–12 nucleotides, which is removed by the flap endonuclease 1 (FEN1). Finally, intervention of the PCNA-associated DNA Ligase I seals the nick [6].

Notably, BER protein components are involved in at least two subpathways, namely Single Strand Break Repair (SSBR) and Nucleotide Incision Repair (NIR) [7]. SSBs are generated by different sources including reactive oxygen species (ROS), radiomimetic drugs, ionizing radiation, topoisomerase-mediated DNA cleavage or they are unavoidable intermediates generated during BER processing. The SSBR pathway initiates through recruitment of the poly(ADP-ribose) polymerase PARP1, which recognizes exposed SSBs and modulates the repair process through enzymatic ADP-ribosylation of protein substrates. Tight connection between BER and SSBR has been highlighted by the observation that many BER proteins (e.g. XRCC1, Polβ) interact with PARP1 [6] and by the fact that PARP1 has been shown to orchestrate the BER processing of uracil and AP-sites [8]. Interestingly, it has been recently demonstrated that APE1 has glycosylase-independent NIR activity on particular modified bases, see below. Although very intriguing, the physiological impact of the NIR pathway is still under investigation, as within the intracellular milieu the presence of specific DNA glycosylases would likely dampen the efficiency of the NIR process on DNA.

Overall, BER is currently regarded as a dynamic intertwining of different enzymes and auxiliary proteins that operate in a highly coordinated manner to allow temporal and spatial modulation. The relevance of this coordination is remarked by several observations, which still deserve further studies:

- imbalanced expression of BER components has been linked to genomic instability. In particular, overexpression of core elements of the pathway is a hallmark of cancer progression and resistance to therapy. Increased expression of a single BER factor may result in competition or in excessive enzymatic activity, which is not compensated by equimolar amounts of other BER proteins. This has been formally demonstrated, in the case of APE1 [9–11] and Polβ [12].
- abortive intermediates of the pathway are intrinsically cytotoxic since unprotected intermediates (e.g. SSBs) are much more toxic than the initial damaged base [13]. Therefore, the fine-tuning and coordination of the pathway is possibly the result of an evolutionary tradeoff between the rapid repair of mutagenic lesions and the potentially hazardous intermediates that such repair may generate.

In order to explain mechanisms evolved to optimize the repair efficiency of the BER pathway, several models have been proposed including those of the 'passing the baton' and the 'BERosome' [7,8,14]. Despite the apparent divergence amongst models that have been put forward to explain the complexity of BER, each of them probably describes different aspects of a unique and highly dynamic integrated process. However, it is clear how it is modulated through a complex network of more or less stable DNA-mediated or protein-protein interactions, among BER enzymes and non-enzymatic scaffold proteins (e.g. XRCC1, PCNA) and PTMs (Post-translational modifications). Phosphorylation, acetylation, methylation, SUMOylation, as well as ubiquitination of almost every BER component have been suggested to play a role in the modulation of the pathway [7].

An emerging concept in this field is the role of some non-canonical regulatory proteins as BER modulators. Several proteins, apparently unrelated to the pathway, have recently been discovered as novel unexpected coordinators of BER [15]. p53, for instance, has been implicated in the modulation of both APE1 and Polβ [6], whereas our laboratory discovered nucleophosmin (NPM1) as a modulator of the APE1 enzymatic activity [16]. Additional regulation of the BER pathway is also achieved through evolutionarily acquired disordered extensions of some BER components [17,18]. These accessory proteins were proposed to be important for stabilizing large complexes, "repairsome", by providing extended interaction surface area [19].

An interesting link between DNA damage sensing and modulation of BER protein amount has been recently demonstrated. Indeed, BER proteins amount, which is generally abundant with a relatively long half-life, is constantly oscillating in response to the DNA damage load at the steady-state level. This equilibrium is strictly controlled by the ubiquitin-proteasome system [4]. It is possible that the high level of them (such as APE1 and Polβ) observed in several tumors is the result of perturbations of this equilibrium. Understanding these aspects will shed light on the role of BER proteins in cancer development.

2. NIR activity of APE1 on non-canonical substrates

In the last decade, Nucleotide Incision Repair (NIR) pathway has been described as a new function of APE1 which works as back up of BER pathway ensuring a correct removal of damaged bases as a result of oxidative stress [20–25]. NIR activity by APE1 consists of an incision at

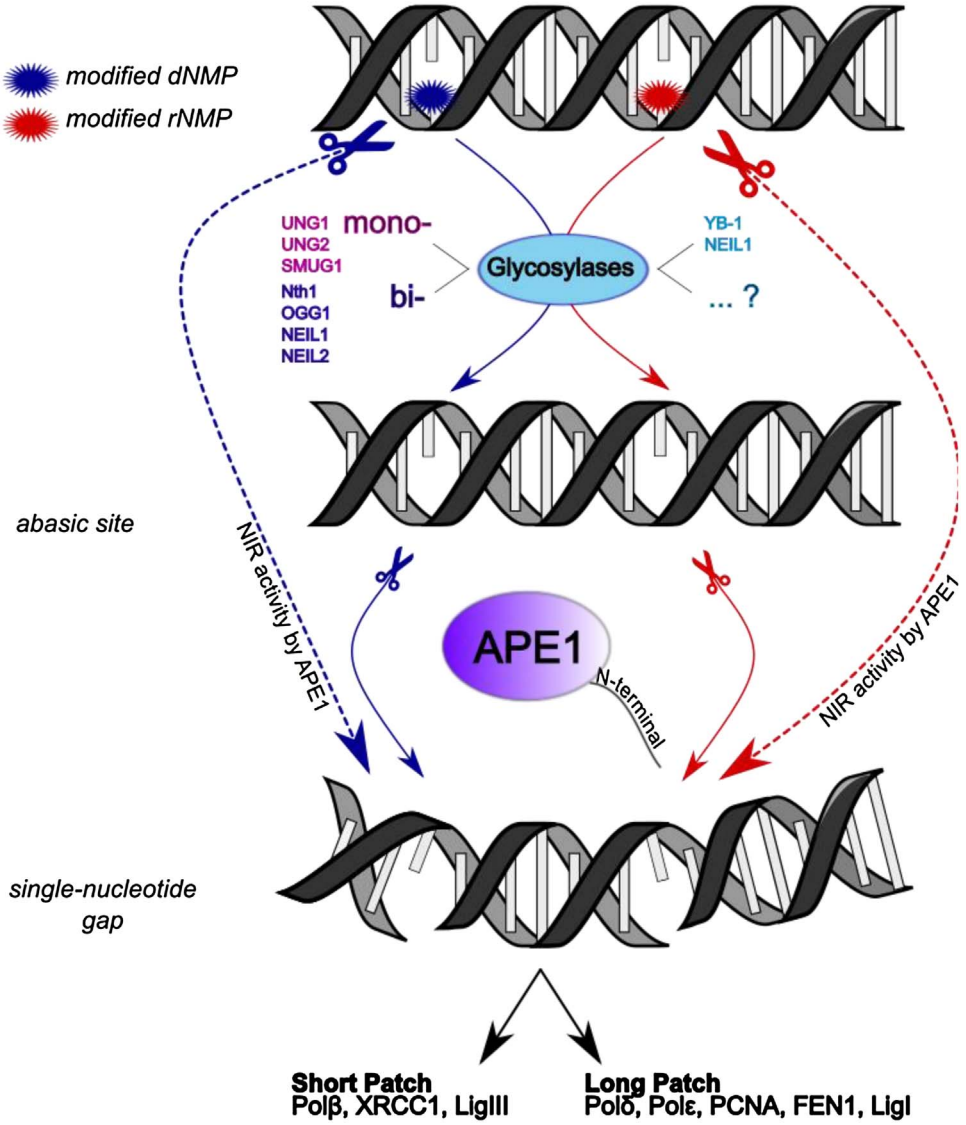

Fig. 2. Schematic representation of consecutive BER steps during the repair of different DNA lesions. BER is endowed to repair different lesions including modified dNMPs (blue star) and modified rNMPs (red star). Different types of mono- and bi- functional glycosylases exist in order to recognize damaged dNMPs, such as oxidized dNMPs, uracil, etc., and generate deoxy-abasic sites, which are subsequently processed by APE1. At the same way, an oxidized rNMPs, embedded in DNA, could be recognized by specific glycosylases such as YB-1 and NEIL1, and be converted in ribo-abasic sites, efficiently processed by APE1. For both types of damages, APE1 possesses a NIR activity in which APE1 cleaves directly the lesion bypassing the glycosylases action. BER pathway is finally terminated by different specific enzymes depending on whether the Short Patch or Long Patch pathways.

the 5′ next to a oxidatively damaged base in a DNA glycosylase–independent manner, providing a proper 3′-OH group for further processing [20]. After the removal of the dangling damaged nucleotide by a flap endonuclease [26,27], DNA backbone can be efficiently repaired by a DNA polymerase. In this way, the NIR action avoids the generation of potentially toxic AP-intermediates [28]. Several uncanonical substrates are processed by APE1 through NIR activity, including 5,6-dihydro-2′-deoxyuridine (DHU) [20,28], 5,6-dihydrothymidine (DHT) [20,28], 5-hydroxy-2′-deoxyuridine (5OHU) [20,28], 5-hydroxy-2′-deoxycitidine (5OHC) alpha-2′-deoxynucleosides (αdA, αdT and αdC) [20,28–31], the majority of which are generated under ionizing radiation (IR) and exposure to certain drugs. This non-canonical activity of APE1 could explain how, on the contrary to what is observed in APE1 deficient cells [32–34], the lack of DNA glycosylases does not sensitize cells or mice to oxidative agents and IR [35–37]. Although NIR pathway plays an important role as a back-up of BER, experimental working conditions, such as salts and pH conditions, seem to affect the NIR function of APE1. The optimal conditions for NIR activity are very similar to those required for the 3′– > 5′ exonuclease activity by APE1, characterized by a pH around 6.4–6.8, and a KCl concentration of 50 mM [20]. Moreover, NIR is more active to 100-fold lower $MgCl_2$ concentration compared to that of the canonical AP-endonuclease activity [20]. Interestingly, the N-terminal domain of APE1 deputed to modulate protein-protein interaction and indispensable to redox activity but not AP-endonuclease activity [38], is contrarily essential for the NIR activity; indeed the lack of the first 33 N-terminal aminoacids of the protein produces a 20-fold-decrease of APE1 NIR activity [20]. Moreover, Timofeyeva et al. demonstrated that Lysine in position 98 contributes significantly in the 5′-phospodiester bond hydrolysis of DNA substrate, but not in the dissociation of the enzyme-product complex [21]. Interestingly, the substitution of this amino acid influences the APE1 NIR activity more than BER, demonstrating that the APE1 active site involved in NIR and BER pathways is the same, but different conformational requirements are responsible for APE1 NIR or BER activities [21].

3. Unusual involvement of BER enzymes in the regulation of gene expression

Generation of single base modifications is not only a harmful DNA modification caused by genotoxic agents but it may have important regulatory functions also at the basis of epigenetic mechanisms. For instance, 5-methylcytosine and Uracil, which are enzymatically generated during epigenetic regulation of gene expression and in antibody diversification processes, respectively, are the most well-known examples of this phenomenon. Very recently, a number of studies pointed to 8-oxoG (8-oxo-7,8-dihydroguanine) modification as a new epigenetic mark with a relevant role in the control of gene expression [39]. Generation of 8-oxoG on different promoters regulates the activity of

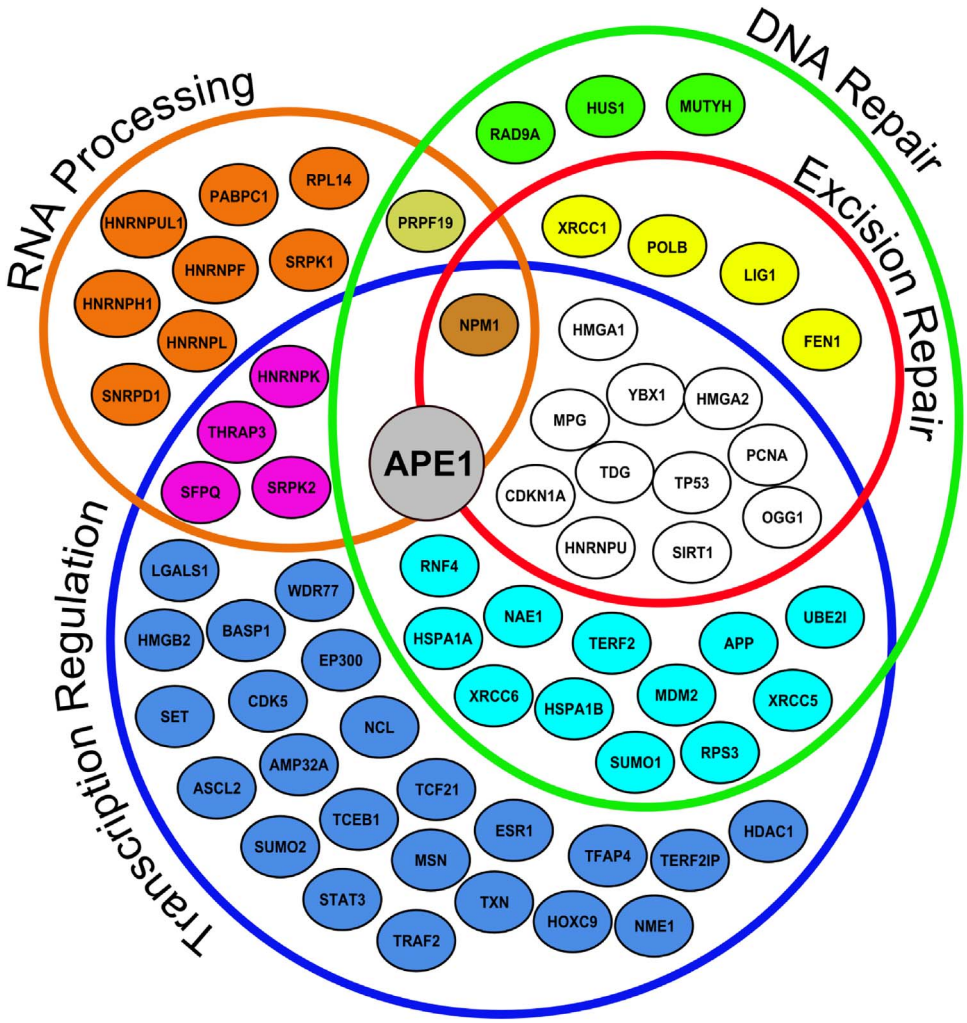

Fig. 3. APE1 protein interactors are highly interconnected.
The top four functional annotation clusters of APE1 interactors identified by DAVID enrichment analysis based on gene ontology terms of biological processes. Protein interactors that are found in more than one cluster are colored accordingly.

several transcription factors (e.g. Hif1α, STAT1, NF-κB and MYC) in conjunction with OGG1 protein and, in turn, regulates the expression of important genes involved in cancer development, such as VEGF [40], SIRT-1 [41] and inflammatory genes such as TNF-α and CXCL2 [42]. Interestingly, this last role of OGG1 on inflammatory genes may explain the reduced inflammatory response in $Ogg1^{-/-}$ mice [43].

Canonical epigenetic mechanisms seem to crosstalk with new DNA damage epigenetic marks. In fact, it has been recently shown that demethylation of histone H3 at Lys9 by LSD1 de-methylase, through generation of H_2O_2, promotes oxidation of estrogen-receptor responsive promoters in breast cancer cell lines with a consequent OGG1-dependent promoter activation [44]. In a similar way, also Myc-induced, retinoic acid-induced and androgen receptor-induced transcription has been proved [45–47] demonstrating that the transcriptional function of OGG1 is a much more general non canonical function than previously thought. OGG1, in complex with 8-oxoG free base, has been recently suggested to play a role in signal transduction as a new Guanine Exchange Factor (GEF) for Ras, Rac and Rho GTPases [48–50]. These findings open new completely unpredicted perspectives for this unusual DNA-repair enzyme in controlling gene expression.

Another well-known enzyme of the BER pathway having important function in transcriptional regulations is APE1 [11,51]. APE1 plays a role in the regulation of expression of human genes during oxidative stress conditions and it is important for cancer biology, through indirect- and direct-mechanisms [52]. Thus, besides providing a crucial role in the genome stability maintenance, APE1 acts also as a master regulator of cellular response to genotoxic damage via indirect mechanisms. Indeed, by regulating the expression of several tumor related genes, through the stimulation of DNA binding activity of transcription factors such as NF-κB, Egr-1, Hif-1α, Nrf1, APE1 can influence the onset of inflammatory and metastatic progression [11]. Through the redox-mediated activation of NF-κB and Hif1α, for example, APE1 indirectly drives IL-8 and VEGF expression, respectively, thus acting as a key regulator of inflammatory and tumor-associated neo-angiogenesis processes. Moreover, APE1 has been implicated in chemoresistance, considering its ability to stimulate the expression of the multi-drug resistance gene MDR1 through the interaction with Y-box-binding protein 1 (YB-1) [53], and its regulatory abilities on the PTEN tumor suppressor [54,55]. We recently characterized a direct role of APE1 in the transcription of SIRT1 gene through the binding of nCaRE-sequences present on its promoter, demonstrating that BER-mediated DNA repair promotes the initiation of transcription of SIRT1 gene upon oxidative DNA damage [41]. A recently published paper gave a definitive prove to our previous findings and suggested a novel role of APE1 in epigenetic regulation, through modulating in a redox-mediated manner the DNMT1 expression and causing consequent suppression of Oct4 and Nanog expression through specific promoter methylation [56]. The epigenetic role of 8-oxoG modification was further supported by a recent publication showing a regulatory function of BER enzymes on specific gene expression by controlling the topological superstructure of G-quadruplex containing promoters, such as those of *VEGF* and *NTHL1* genes [57], as also discussed in another article of this issue by A. Fleming and C. Burrows. All these studies highlight a new unsuspected function of oxidative DNA lesions as novel epigenetic mechanisms of gene regulation through the action of BER enzymes.

Moreover, despite the previous consistent research into APE1

molecular mechanisms in DNA damage repair, only recently it has been hypothesized that this protein may play an unsuspected though important function in RNA metabolism impacting on the post-transcriptional control of gene expression [17,58,59].

Overall, these pioneering studies hold the promise to change the focus of scientific research from investigating the canonical roles of BER enzymes in DNA repair to the study of their function in gene regulation highlighting the BER peculiarity for eukaryotic cells. Overall, these studies will contribute to open the DNA repair world to RNA universe.

4. BER enzymes and RNA metabolism: unexpected findings from interactomics studies

Emerging evidences have pointed out that DNA damage repair and RNA metabolism are more closely related than previously thought. In addition, ncRNAs and miRNAs act at multiple levels of DNA damage response regulation and have been proposed as novel anticancer therapeutic targets [59]. DNA repair enzymes, in particular a large cohort of BER proteins, have been implicated in RNA metabolism and in transcriptional regulation of genes suggesting that DNA damage repair and RNA pathways are tightly inter-regulated [59].

A number of recent interactomic studies significantly contributed to open new scenarios for the comprehension of non-canonical functions of BER proteins, including understanding their unusual distribution in the nucleolar compartment [17,60,61] (Antoniali et al., submitted). The APE1-interactome network, characterized in part in our laboratory and in different literature works, actually comprises more than 100 different protein species whose functions are related to four biological pathways intimately interconnected: DNA repair, Excision Repair, RNA processing and Transcriptional Regulation (Fig. 3).

Actually, the major focus is on APE1, SMUG1 and PARP1, as prototypical examples [17,59,62–64]. In this review, in particular, we highlight current knowledge of APE1 in controlling RNA metabolism. Interested readers are referred to the aforementioned references for further details about SMUG1 and PARP1.

5. BER enzymes and miRNA regulation: a new paradigm in gene expression?

We recently found that many ncRNAs, few miRNAs and some functional RNAs are directly bound by APE1 in cancer cells (Antoniali et al., submitted). Furthermore, preliminary analysis from our laboratory indicated a potential role of APE1 in miRNA biology through an effect on the Microprocessor complex. These observations prompt a new model that links DNA damage responses and the modulation of target genes, and highlight how APE1 may regulate gene expression through its direct binding and/or processing of specific RNA. Therefore, studying APE1 new function in miRNAs processing may represent a novel field of investigation in cancer biology, which may be linked to its role during cancer progression.

MicroRNAs (miRNAs) are small non-coding RNA that function as a guide in RNA silencing of most protein-coding transcripts thereby being critical regulators of nearly all physiological and pathological processes. As a consequence, a delicate temporal/spatial balance between miRNAs and their targets is central to achieve the appropriate biological outcome and, it is not surprising, that any deregulations of miRNA biogenesis and expression have been linked to a broad spectra of pathological features of human diseases including cancer [65]. Understanding whether APE1 regulation of target genes of genotoxic response, could be mediated through miRNA processing will definitively increase our knowledge regarding its role in tumor progression and chemoresistance.

An increasing number of evidences indicate that miRNA post-transcriptional maturation, rather than transcription, is often perturbed in cancer. The accumulation of miRNAs precursor forms and the corresponding depletion of mature forms have been evidenced in human cancers compared to normal tissue [66], strongly indicating that the impairment of key steps in miRNA biogenesis could be the underlying cause. RNA binding proteins (RBPs) are crucial components in the determination of miRNA function and stability, as they control different steps of miRNA biogenesis, localization, degradation and activity.

Mechanisms regulating mammalian miRNA biogenesis are quite complex and comprise a series of biochemical steps converting a primary miRNA transcript (pri-miRNA) into a biologically active mature form [65,67]. The canonical miRNA biogenesis pathway is characterized by two subsequent ribonuclease reaction steps. First, in the nucleus, where the transcription by RNA polymerase II takes place, pri-miRNAs are recognized and cropped into hairpin-structure precursors (pre-miRNAs) by the Microprocessor complex minimally composed by the nuclear RNase III Drosha and the RNA binding protein DGCR8 (DiGeorge critical region 8)[68]. Subsequently, pre-miRNAs are actively exported by Exoportin-5 in the presence of its Ran-GTP co-factor into the cytoplasm [69] where a second-round ribonuclease reaction mediated by another RNase III enzyme, Dicer, generates a mature ~22nt duplex miRNA [70]. Only one strand of this miRNA duplex is loaded onto the RNA-induced silencing complex (RISC) whereas the other is usually degraded. Mature miRNAs function as guides by base pairing the RISC complex to its target mRNAs, whereas the Argonaute (Ago) family proteins serve as effectors by recruiting factors that induce translational repression, mRNA deadenylation and mRNA decay [71].

In the last decade, as scientists began to characterize a larger fraction of miRNA genes, it appeared that the complexity and pleotropic nature of miRNAs have been underestimated [72]. The multiple steps of miRNA maturation could potentially provide a variety of molecular options revealing a complex dynamic in miRNA processing. Recent studies have pointed out alternative pathways for maturation of certain miRNAs that deviate from the canonical miRNA biogenesis pathway bypassing one of the RNase III cleavage steps [72–74]. This is the case of the mirtrons, pre-miRNA hairpins generated by splicing and debranching of short hairpin introns, and other small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), tailed endogenous shRNA, long hairpin RNAs (hp-RNAs) derived from short interspersed nuclear elements (SINEs) that bypass the cleavage step of Drosha [75–77]. To date, miR-451, an erythropoietic miRNA conserved in vertebrates, is instead the only Dicer-independent miRNA identified [78].

Furthermore, there is still a great uncertainty regarding the exact composition of the miRNA-processing complex. Although the core components, Drosha and DGCR8 are required for the biogenesis of almost all miRNAs, the Drosha complex contains numerous auxiliary factors including the DEAD-box RNA helicase p68 (DDX5) and p72/p82 (DDX17) which selectively promote the activity of Drosha processing over certain pri-miRNAs. Dicer is instead associated with TAR RNA-binding protein (TRBP) and the kinase R-activating protein (PACT) [67]. Several studies have further revealed a number of further RNA binding proteins that are required for miRNA biogenesis. These modulators have been shown to positively or negatively regulate miRNA biosynthesis by directly binding miRNA terminal loop region, thus affecting Drosha and/or Dicer interaction with pri-miRNA or pre-miRNA. More importantly, deregulation of RBPs expression and activity has been linked to several malignancies. Among the panel of factors identified for example: DDX1, BRCA, ARS2, DR5, ADAR1, hRNP A1, KSRP, Lin 28, SMADs, YAP, ERα, ERβ, wtp53 and mutant p53 [79,80]. Of note, included in this list of RBPs, there are few DNA damage response (DDR) proteins [81].

Remarkably, several groups pointed to a specific miRNA-RBP interplay in response to external stimuli including DNA damage. Different enzymes involved in DDR have been shown to participate in miRNA processing and maturation (Fig. 4). Up-regulation of specific miRNAs such as miR-16-1, miR-143, miR-206 and miR-145 has been shown to be induced in a p53-dependent manner after DNA damage. p53 is able to interact with the Drosha complex through p68 increasing pri-miRNA biogenesis. Interestingly, transcriptionally inactive p53 mutants disrupt

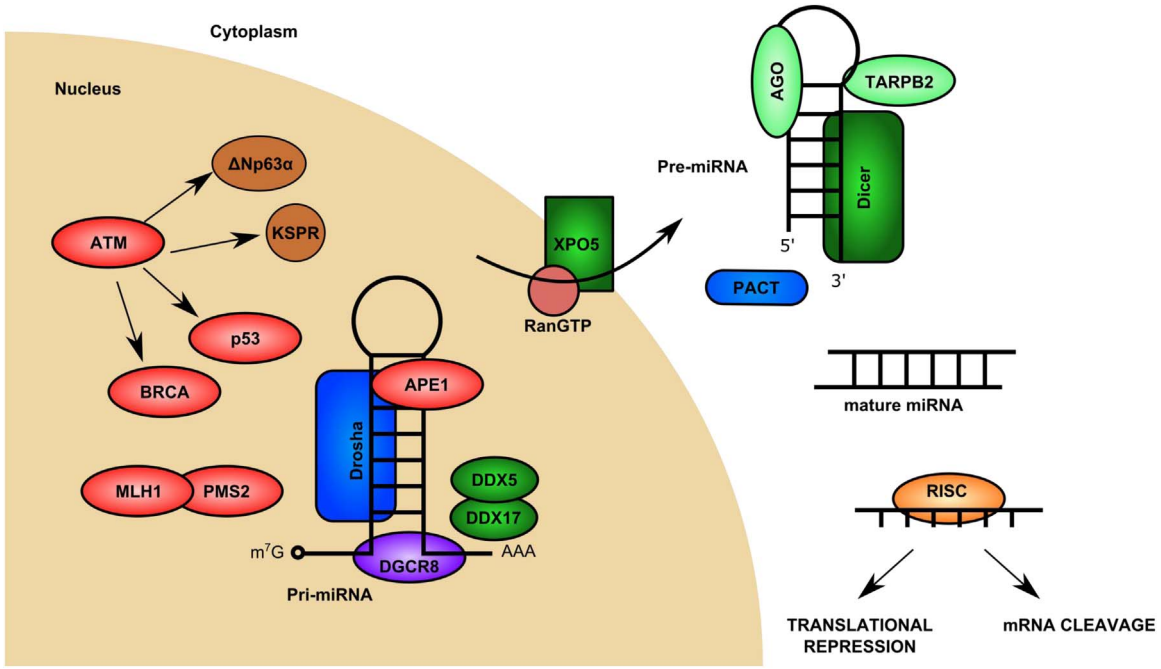

Fig. 4. Is BER involved in miRNA processing?
Regulation of miRNA biogenesis pathway by DNA damage response (DDR) proteins. Several DNA repair enzymes influence the processing of a subset of miRNA by recognizing specific miRNA features or by associating with key components of the miRNA maturation pathway. Red boxes indicate DDR proteins involved in miRNA biogenesis.

Drosha and p68 complex suppressing miRNA processing activity of Drosha by sequestering p68 cofactor [79,82].

Additionally, BRCA1, a key player in DSBs (Double Strand Breaks) response, has been recently shown to increase the expression of let-7a-1, miR-16-1, miR-145, and miR-34a through a direct binding of p68 RNA helicase in Drosha complex [83].

ATM, a crucial kinase in DDR, regulates miRNA expression in response to DNA damage through the phosphorylation of different targets including, KSPR [84], p53 [84,85], ΔNp63α [86] and BRCA1 by indirectly promoting the processing of a subset of pri-miRNAs [83].

MMR pathway can also mediate miRNA processing. The heterodimer MLH1-PMS2 (MutLα) has been shown to positively regulate the processing of miR-422a and other miRNAs by specifically stimulating the Drosha/DGCR8-catalyzed processing of pri-miRNAs to pre-miRNAs [87].

Different studies have also suggested a possible involvement of BER proteins in miRNA processing, particularly those proteins found to be multifunctional enzyme with unique features such as: YB-1, Poly(ADP-ribose) polymerases (PARPs) and APE1.

YB-1 is a multifunctional protein participating in a variety of DNA/RNA-dependent events such as DNA replication and RNA-processing events including mRNA transcription, splicing, translation and stability. Interestingly, in light of its interaction with different DNA repair enzymes, it is considered as a non-canonical BER protein [88]. In addition, genome-wide analysis of YB-1-RNA interaction in glioblastoma cell line unraveled a novel role of YB-1 in the regulation of miRNA biogenesis during tumorigenesis. Wu et al., recently demonstrated that YB-1 interaction with the loop region of pre- and pri-miR-29b-2 interferes with Drosha and Dicer cleavage step. Interestingly, down-regulation of miR-29b-2 expression as a consequence of this missed recruitment of Drosha and Dicer to miR-29b-2 precursor due to YB-1 blockage, has crucial implication in glioblastoma proliferation [89].

Recent data suggest that miRNA biogenesis can be also modulated by post-translational modifications of each member of the Argonaute family by PARPs [90,91]. Indeed, poly(ADP-ribose) modification of key RNA regulatory proteins has been documented for every step of RNA metabolism including miRNA biogenesis. There are five specific PARPs that can be considered RBPs because of the presence of RNA binding domain in their sequence (i.e. CCCH zing finger and RRMs, RNA recognition motif): PARP7, PARP12, PARP13, PARP10 and PARP14. In particular, it has been observed that, upon stress condition, an increased PARP13 activity near the Argonaute/miRNA complex might result in the disruption of the electrostatic interaction between miRNA and its mRNA target with a consequent mRNA cleavage relief [91].

APE1 possible involvement in miRNA biogenesis was previously only speculative based on different findings. It has been demonstrated that APE1 can directly bind, *in vitro,* structured RNA molecules through its 33 amino acids N-terminal domain [92] and, more interestingly, that APE1 is endowed with RNA-processing activity over single-stranded RNA, regulating for example c-Myc mRNA level and half-life in tumor cells [93] and, of note, it can cleave pre-miR10b and pre-miR-21 interfering with Dicer processing *in vitro* [94]. In addition, to further support APE1 involvement in RNA processing it has been also demonstrated its 3′-RNA phosphatase and 3′-exoribonuclease activities [95].

As a supplementary indirect observation, it has been recently found that APE1 down-regulation is associated with alteration in miRNAs expression, which are involved in pathways relating to developmental and regulation of cellular processes, cell signaling and cancer [96].

Very recently, in order to improve our knowledge on APE1 possible involvement in miRNA maturation, by using a combination of different unbiased high-throughput approaches at the transcriptomic and proteomic levels, we demonstrated that APE1 is involved in miRNome regulation by acting on early phases of miRNAs processing (Antoniali et al., submitted). We found more than 1000 APE1-bound RNA among which many ncRNAs and notably pri-miRNAs that are directly bound by APE1 in cancer cells. In particular, we showed that APE1 endonuclease activity over pri-miR-221/222 mediates the regulation of the tumor suppressor PTEN, a known target of these miRNAs [97]. Moreover, we evidenced, for the first time, that APE1 associates with the Drosha microprocessor complex during oxidative stress suggesting a possible contribution of APE1 in RNA-decay pathways controlling miRNAs precursors stability in the genotoxic cell response. Therefore, since APE1 is an interacting partner of Drosha and of p53 [98] and hRNPA1 (Antoniali et al., submitted), both proteins described as modulator of Drosha-mediated cleavage, we may speculate that APE1 endoribonuclease activity is part of inducible mechanisms regulating

the processing of miRNA biogenesis in the nucleus especially during oxidative stress or genotoxic damage. This is further support by the observation that oxidative stress promotes APE1/Drosha interaction and that APE1-kd is associated to increase oxidation levels of precursors miRNAs. These findings, together with previous data that APE1 silencing is associated with increased RNA oxidation [99], would support a major role of APE1 in the RNA-decay mechanisms of precursors pri-miRNAs.

Of note, not only RNA-binding factors can influence miRNA processing, but also post-transcriptional modification such as RNA-editing may change both maturation and expression of miRNAs. Adenosine to inosine (A-to-I) RNA-editing within the hairpin region of miRNA precursors is among the major effective mechanism described to alter the primary sequence of RNA. A-to-I miRNA editing is mediated by adenosine deaminases acting on RNA (ADAR) protein, ADAR1 and ADAR2, having consequences in miRNA biogenesis both at the level of Drosha and Dicer [100]. Recently, Wang et al. demonstrated that also oxidation represents another miRNA post-transcriptional modification with remarkably pathological outcomes. They proposed a new model in which ROS may modulate cellular events by oxidatively modifying miRNAs, as in the case of miR-184, which after oxidation changed its binding proprieties from native targets to new ones [101].

In light of our previous data, demonstrating that APE1 has endoribonuclease activity over abasic RNA [99], we should therefore reinterpret the roles of APE1 in modulating cellular responses to genotoxic stresses and in the pathogenesis of human diseases, taking into account the new role of this multifunctional protein in RNA biology. Our data demonstrate that APE1 is involved in specific miRNA processing and highlight a new mechanism of miRNA regulation with profound relevance in tumor biology.

In conclusion, recent advances in miRNA maturation pathways have demonstrated that the multiple stages of miRNA biogenesis could serve a multitude of regulatory options in control of miRNA-dependent gene regulation. These findings have changed the conventional concept of miRNA processing and have shown an additional level of complexity in the miRNA network. miRNAs are often deregulated in human pathologies including cancer; frequently this change is a consequence of impaired transcription rate and/or miRNA processing. RBPs and their interacting modulators mostly account for these observed changes in miRNAs processing and activity.

Further investigations on the additional mechanisms that control the processing of miRNAs under various cellular conditions and the role of DNA repair proteins will extend our knowledge on miRNA function in both physiological and pathological processes. These findings will also offer a molecular basis for diagnostic and therapeutic strategies based on miRNA biology.

6. New insights about BER involvement into removal of modified ribonucleotides embedded in DNA

A “new type of damage”, abundantly explored in the very last years, is the presence of ribonucleotides monophosphate (rNMPs) within genomic DNA. It has been studied how the additional presence of a reactive 2′-hydroxyl group on the sugar ring may: i) alter the DNA physical properties, reducing its elasticity [102,103] and structure in a sequence dependent manner [104–108]; ii) induce DNA replication or transcription arrest making the DNA backbone prone to hydrolysis triggering a persistent genomic instability [103,108,109]. This incorporation occurs quite frequently into the cell [110,111], potentially during every DNA replication reaction [110]. It has been estimated that a few thousands of rNMPs are embedded in budding yeast genome [110] and over a million in mouse genome [112]. The higher amount of the cellular pool of ribonucleotides (rNTPs), compared to their corresponding deoxyribonucleotides (dNTPs) [110] counterparts, combined to an incomplete elimination of RNA primers used in the generation of Okazaki fragments [113] and an imprecise 3′-exonucleolytic proofreading activity of replicative DNA polymerases [113–117] are some of the major causes of the rNMPs incorporation into genomic DNA. Moreover, another potential significant, yet poorly characterized, source of rNMPs incorporated in DNA is oxidative stress. Specifically, ROS, among which the hydroxyl radical ‘OH is the most reactive, can attack all components of DNA, including the deoxyribose giving rise to ribose [118], both *in vitro* and *in vivo*. Moreover, oxidation can occur not only in DNA but also in the nucleotides pool and then rNMP may be incorporated into neo-replicated DNA. During last years, several approaches have been developed [119], including Ribose-seq, in order to map rNMPs sites into genomic DNA [111]. By using these methods, it has been discovered that the incorporation of rNMPs has a widespread but not random distribution in chromosomal DNA of budding and fission yeast [111] and their number, per nuclear chromosome, seems to be proportional to the chromosome size [111].

It is imperative that mis-incorporated rNMPs should be efficiently and rapidly repaired by the cell, otherwise the effect on genome stability and cell survival can be disastrous. Processing of single rNMPs embedded in DNA is guided by the Ribonucleotide Excision Repair (RER) pathway in which the leading initiating enzyme is RNase H2 [120] deputed to incise the phosphodiester linkage at 5′ end of rNMPs [121]. Conditions in which human *RNASEH2* is mutated, are associated with a neurological auto-inflammatory childhood disorder named Aicardi-Goutières Syndrome (AGS), characterized by an over production of IFNγ [122–124]. It has been demonstrated that AGS patients, having altered RNase H2 activity, may accumulate rNMPs in DNA, which could induce a chronic, low-level DNA damage response signaling that stimulates innate immune pathways [125]. Moreover, RNase H2-null murine embryonic fibroblasts (MEFs) accumulate over 1 million rNMPs in their genomic DNA, activating a p53-dependent damage response, whereas null-RNase H2 are embryonic lethal [121]. While, under normal physiologic conditions, the involvement of functional RER pathway in repairing the rNMPs incorporated in DNA is known [112,120] information is scanty about possible back-up mechanisms, provided by other DNA-repair pathways, when RER is functionally impaired. In the absence of RNase H2, Topoisomerase I cleavage [126] followed by nick processing by Srs2–Exo1 can remove some rNMPs [127,128]. Paired and mispaired rNMPs in DNA can also be targeted by the nucleotide excision repair (NER) in bacteria [129] but likely not in mammalian cells [130], and by the mismatch repair (MMR) systems [131,132]. About this last pathway, it was hypothesized the incorporation of ribonucleotides into mismatching damaged DNA could be interpreted as a putative “benefic” role, verifiable in a signal for the activation of MMR pathway [133].

However, nothing is currently known about the mechanisms, if any, responsible for repairing modified rNMPs embedded in DNA such as abasic or oxidized rNMPs. Future studies are required to address this issue.

7. Open questions and future perspectives

Based on the observations described so far, many open questions remain to be addressed:

- Considering that many BER enzymes are catalytically active in many different RNA substrates, are their main functions linked to RNA-decay processes or in the editing processes?
- May the role of BER enzymes, both mRNA and ncRNA, affect the protein translational machinery thus contributing to gene expression regulation through post-transcriptional mechanisms?
- It is unknown whether abasic RNA may derive from enzymatic processing of modified (i.e. oxidized, alkylated) RNA. Are there specific BER glycosylases responsible for recognition and processing of these substrates?
- Is the BER involved in processing modified ribonucleotides embedded in human genome, which are not repaired through canonical

RER?

- Based on the emerging role of 8-oxoG as a new epigenetic mark, do BER proteins work as novel epigenetic regulators?
- Pondering the many evidences about the relevance of non-canonical function of BER proteins, should we reinterpret the overall biological function of BER and their role in cancer and neurodegeneration?

Answer to these questions may help us to better understand the role of BER pathway in different pathological processes from neurodegeneration to cancer development moving from an old tolemaic and reductive vision, which considers BER only a mere DNA repair pathway, to a more galileian theory which regards also its essential function in RNA biology and thus may explain its unpredicted role in gene expression.

Conflicts of interest

None declared by the author.

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Inhibitors of the Apurinic/Apyrimidinic Endonuclease 1 (APE1)/Nucleophosmin (NPM1) Interaction That Display Anti-Tumor Properties

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The apurinic/apyrimidinic endonuclease 1 (APE1) is a protein central to the base excision DNA repair pathway and operates in the modulation of gene expression through redox-dependent and independent mechanisms. Aberrant expression and localization of APE1 in tumors are recurrent hallmarks of aggressiveness and resistance to therapy. We identified and characterized the molecular association between APE1 and nucleophosmin (NPM1), a multifunctional protein involved in the preservation of genome stability and rRNA maturation. This protein–protein interaction modulates subcellular localization and endonuclease activity of APE1. Moreover, we reported a correlation between APE1 and NPM1 expression levels in ovarian cancer, with NPM1 overexpression being a marker of poor prognosis. These observations suggest that tumors that display an augmented APE1/NPM1 association may exhibit increased aggressiveness and resistance. Therefore, targeting the APE1/NPM1 interaction might represent an innovative strategy for the development of anticancer drugs, as tumor cells relying on higher levels of APE1 and NPM1 for proliferation and survival may be more sensitive than untransformed cells. We set up a chemiluminescence-based high-throughput screening assay in order to find small molecules able to interfere with the APE1/NPM1 interaction. This screening led to the identification of a set of bioactive compounds that impair the APE1/NPM1 association in living cells. Interestingly, some of these molecules display anti-proliferative activity and sensitize cells to therapeutically relevant genotoxins. Given the prognostic significance of APE1 and NPM1, these compounds might prove effective in the treatment of tumors that show abundant levels of both proteins, such as ovarian or hepatic carcinomas.

Key words: APE1; NPM1; protein/protein interaction; small molecule; combination therapy

INTRODUCTION

Targeting DNA repair pathways to improve tumor therapy is currently one of the most active topics in cancer research. Many compounds targeting different DNA repair components are currently undergoing clinical and pre-clinical investigation as promising molecules that display either a selective cancer killing action or, more often, that improve sensitivity to traditional therapy [1–3].

The human apurinic/apyrimidinic endonuclease 1 (APE1) is a pivotal DNA repair protein, being a central enzyme to the base excision repair (BER) pathway. As

Abbreviations: APE1, apurinic apyrimidinic endonuclease 1; BER, base excision repair; E3330, (2E)-3-[5-(2,3-dimethoxy-6-methyl 1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid; HTS, high-throughput screening NPM1, nucleophosmin; PLA, proximity ligation assay; MMS, methyl-methanesulphonate; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; SPR, surface plasmon resonance; TNF-α, tumor necrosis factor-α.

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the main abasic endonuclease in mammalian cells, this protein is essential for embryonic development [4,5]. In addition, APE1 acts as a master regulator of cellular transcription, by modulating in a redox-dependent and independent fashion the DNA binding activity of several cancer-related transcription factors (including NF-κB, Egr-1, p53, HIF-1α among others) [6]. Aberrant subcellular localization, expression levels, and post-translational modification patterns of APE1 have been linked to increased tumor aggressiveness and decreased differentiation, as well as to the onset of chemo- and radio-resistance in different kinds of cancer [7–12]. In light of the association between APE1 and cancer, several laboratories over the last decade have developed strategies to target either its endonuclease activity or its redox function by means of small molecule inhibitors [3,13–15]. Since APE1 is a ubiquitous protein [10], it is not clear whether these approaches could achieve specificity of action in the contest of a systemic administration of the APE1 inhibitor.

We previously reported and characterized the molecular association between APE1 and nucleophosmin (NPM1) [16–18], a nucleolar phosphoprotein involved in tumorigenesis, either as a proto-oncogene or as a tumor suppressor, in a context-dependent manner [19,20]. The interaction with NPM1 modulates several functions of APE1: it promotes APE1's accumulation within nucleoli, stimulates its endonuclease activity, and, likely, regulates its post-translational modifications and protein interaction network, by masking the unstructured N-terminal domain of the protein [8,16,21]. Very recently, we showed that NPM1 is involved in the functional modulation of the BER pathway in cells through direct interaction with APE1 and stimulation of its AP-endonuclease activity [22]. Accordingly, the occurrence of an aberrant cytoplasmic APE1/NPM1 association, observed in acute myeloid leukemia (AML) patients bearing a NPM1 mutation (NPM1c+) [20,23], leads to an impairment of the BER pathway and to an increased sensitivity to genotoxins [22]. Moreover, the expression of an APE1 mutant unable to stably interact with NPM1 is linked to a strong reduction in the rate of cellular proliferation [21]. Altogether, these observations suggest that a functional APE1/NPM1 interaction plays a pivotal role in tumor cell proliferation and cell response to genotoxins.

NPM1 overexpression is considered a prognostic marker of recurrence and progression in solid tumors [19,24,25]. Furthermore, altered expression of APE1 has been linked to progression of hepatocellular and ovarian carcinomas [7,9]. Interestingly, in ovarian cancer specimens [25], as well as in hepatic carcinoma cell lines (unpublished data), we detected a positive correlation between NPM1 and APE1 expression levels and the aggressiveness of the malignant phenotype, pointing to these proteins as negative prognostic markers in these pathologies. Such observations suggest that ovarian and hepatic tumors expressing higher amounts of APE1 and NPM1 might display an increased APE1/NPM1 interaction, which may positively impact on tumor cell proliferation and anticancer agent resistance. Interfering with the APE1/NPM1 association, therefore, might prove effective in directly targeting tumor cell proliferation rate and/or in sensitizing them to DNA damaging agents. In addition, this approach might prove more specific and efficient toward cancer cells over-expressing both APE1 and NPM1, overcoming potential systemic toxicity problems.

Here, we describe the discovery, through high-throughput screening (HTS), of small molecules able to interfere with the APE1/NPM1 association. We made use of the AlphaScreen® technology to screen several commercially available small molecule libraries in order to identify, for the first time, a set of molecules that target the APE1/NPM1 interaction. Among the positive hits, we detected known bioactive compounds with novel interesting anti-tumor properties, such as genotoxin-sensitizing and anti-proliferative activities. This study opens new perspectives to target cancer cell proliferation and therapy resistance, while also providing new tools to investigate thoroughly the biological relevance of the APE1/NPM1 association in experimental models and during tumorigenesis.

MATERIALS AND METHODS

Pilot Libraries of Bioactive Compounds

The screening collection included the following libraries with number of compounds in parentheses: FDA Pharmaceutical Collections (2,816), MicroSource Spectrum collection (1,408), Tocris/TimTec (1,395), and bioactive compounds from Sigma–Aldrich LOPAC1280 (1,280), FDA-Tocris-KU-DP (1,376), Prestwick (1,120), BU-GP-BioMol (1,302), Kinacore (2,037), and NCGC chemistry analogues.

AlphaScreen®-Based High-Throughput Screening Assay

Assay buffer consisted of 20 mM potassium phosphate pH 7.0, 50 mM NaCL, 5 mM $MgCL_2$, 0.01% Tween-20, and 2 mM DTT. Microplates used were 384- or 1,536-well white solid-bottom type from Greiner Bio-One (Monroe, NC). Glutathione S-transferase-APE1 fusion protein (GST-APE1) and hexahistidine-labeled NPM1 ($His_{(6)}$-NPM1) proteins were expressed and purified to homogeneity as described previously [16]. AlphaScreen® detection was performed with PerkinElmer Glutathione S-Transferase (GST) and Histidine (Nickel Chelate) Detection Kits (Waltham, MA).

Protein–protein interaction assessment for GST-APE1 and $His_{(6)}$Tag-NPM1 was initially conducted in 384-well plates. The optimized assay was further miniaturized in 1,536-well plates where, briefly, 3 μL of GST-APE1 (125 nM final) was dispensed by BioRAPTR (Beckman Coulter, Fullerton, CA) flying reagent dispenser, followed by addition of 23 nl of

DMSO solution of compound library members (final DMSO concentration was 0.7% [v/v]) achieved by a Kalypsys pintool [26]; after compound addition, 1 μL of $His_{(6)}$Tag-NPM1 (200 nM final) was dispensed and the assay plates were incubated for 20 minutes at room temperature. For detection, 1 μL of 20 μg/mL Glutathione donor/Ni^{2+} chelate acceptor AlphaScreen® bead mix was added. Plates were briefly centrifuged and incubated for 20 min at room temperature. The AlphaScreen® chemiluminescence signal was measured with an EnVision multilabel plate reader (PerkinElmer) equipped with a 1,536 Plate HTS AlphaScreen® aperture (80 ms excitation time, 240 ms measurement time). The signal was compared with that of DMSO-containing control samples; importantly, DMSO had a negligible effect on the assay signal.

Cell Culture, Chemicals and Viability Assays

HeLa, Huh7, and MCF7 cells from ATCC (Manassas, VA) were grown in DMEM (Invitrogen, Milan, Italy). TOV-112D, HCC70 and Ovcar5 cells (from ATCC) were cultured in RPMI 1640 (EuroClone, Milan, Italy), respectively. JHH6 cells (from Health Science Research Resources Bank) [27] were cultured in William's Medium (Sigma, Milan, Italy). OCI/AML cells were obtained and cultured as previously described [22]. Media were supplemented with 10% (v/v) fetal bovine serum (FBS) (EuroClone), 100 U/mL penicillin, and 100 μg/mL streptomycin sulphate. Screening compounds were dissolved in DMSO as 10 mM stocks. MMS was from Sigma–Aldrich and bleomycin sulphate was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

For viability measurements 4-12×10^3 cells were seeded in 96-well plates; 24 h later cells were treated with the indicated compound and cell viability was assessed at the indicated time points through the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay – Promega, Milan, Italy) as per manufacturer's instructions. For MMS-combined treatments cells were pre-treated with the selected protein–protein interaction inhibitor as indicated, incubation was then carried out for further 8 h with increasing amount of MMS, in presence of unchanged inhibitor concentration. For combined treatments with bleomycin 3.0×10^4 cells were seeded into 12-well plates; 24 h later cells were pre-treated for 8 h with the APE1/NPM1 inhibitors at the indicated concentration and then incubation was carried out for 1 h in presence of bleomycin and unchanged compound concentration. Medium was replaced and viability measured 48 h later by Trypan Blue exclusion. All viability assays were performed in triplicate or quadruplicate and reproduced at least twice in independent experimental sessions.

Immuno-Fluorescence and Proximity Ligation Assay (PLA)

Immuno-fluorescence procedures were carried out as described earlier [21]. To monitor the interaction between APE1 and NPM1 in living cells, we used the in situ Proximity Ligation Assay kit (Olink Bioscience, Uppsala, Sweden). This assay detects stable, as well as transient interactions by means of a pair of antibodies against the target proteins; short oligonucleotides linked to the antibodies allow a rolling cycle amplification-based detection of the protein–protein interaction. The signal is visualized though hybridization of fluorescent probes to the amplified oligonucleotides and appears as bright spots that are readily detected through confocal microscopy [28,29]. HeLa cells stably expressing a FLAG-tagged form of APE1 [21] were seeded on glass slides, treated with the selected compounds, fixed with 4% (w/v) paraformaldehyde immediately after the treatment and incubated with a FITC-conjugated mouse anti-FLAG antibody (1:200– Sigma) for 3 h at 37°C. Cells were then incubated with a rabbit anti-NPM1 (1:200–Abcam, Cambridge, UK) overnight at 4 °C. PLA was performed following manufacturer's instructions. Technical controls, represented by the omission of either the anti-NPM1 or the anti-FLAG primary antibodies, resulted in the complete loss of PLA signal. Determination and scoring of PLA signals was performed using a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 488-nm argon laser, a 543-nm HeNe laser, and a 63X oil objective (HCX PL APO 63X Leica). At least 35 randomly selected cells per condition were analyzed by sectioning the whole cell height into six focal stacks, which were averaged and combined into a single image. This procedure allowed us to detect the PLA signals present throughout the cell, regardless of their subcellular localization. PLA-spots present in each single cell were then scored using the Blob-Finder software (Center for Image Analysis, Uppsala University, Uppsala, Sweden); anti-FLAG staining for APE1 was used to identify cell nuclei, allowing us to distinguish between nuclear and cytoplasmic interaction signals.

Surface Plasmon Resonance (SPR) Experiments

Real time binding assays were performed on a Biacore T-100 Surface Plasmon Resonance (SPR) instrument (GE Healthcare, Milan, Italy). Recombinant APE1, APE1 NΔ33, APE1 KA and NPM1 were immobilized at similar immobilization levels (~2600, 2400, 2400, and 2800 RU, respectively) on a CM5 Biacore sensor chip in 10 mM sodium acetate, pH 5.5, by using the EDC/NHS chemistry, with a flow rate of 5 μl/min and an injection time of 7 min, as previously described [16]. Binding assays were carried out by injecting 100 μl of analyte, at 60 μl/min, with HBS (10 mM Hepes, 150 mM NaCL, 3 mM EDTA, pH 7.4), 0.1 mM TCEP, 10% (v/v) DMSO as running-buffer. The BIAevaluation analysis package (version 4.1, GE Healthcare), was used to subtract the signal of the reference channel.

In Vivo Assessment of the APE1 Redox Activity

The APE1 redox function was assessed as described in Ref. [27]. Briefly, 1.1×10^4 JHH6 hepatocarcinoma cells were seeded in 96-well plates, 24 h later cells were co-transfected with 78.4 ng of pIL-8 (interleukin 8 promoter-driven firefly luciferase reporter), 1.6 ng of pRL-CMV (*Renilla* reporter, as a reference for transfection efficiency), and 120 ng of pUC9 carrier plasmid, using the Lipofectamine® 2000 Reagent (Invitrogen) as per manufacturer's instructions. One day after transfection cells were pre-treated with the selected APE1/NPM1 inhibitor (10 μM for 5 h), or with (2E)-3-[5-(2,3-dimethoxy-6-methyl 1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid (E3330, Sigma) as positive control (100 μM for 4 h) in serum-free medium and subsequently challenged with 2000 U/ml TNF-α (PeproTech Inc., Rocky Hill, NJ) for further 3 h. The activity of luciferases was eventually measured using the Dual-Glo® Luciferase Assay System (Promega) using a Modulus™ II Microplate Multimode Reader (Turner Biosystems Inc. Sunnyvale, CA).

Colony Formation Assays and Assessment of the Cellular Growth Rate

For colony formation assays 2×10^2 HeLa cells were plated onto 6-well plates; 24 h later cells were exposed to the selected compound(s) and cells were allowed to grow until formation of visible colonies (9–11 days) and stained as described earlier [17]. To test the effect of chronic exposure to the APE1/NPM1 inhibitors, medium with the indicated amount of fresh compound was replaced every three days. In the experiments combining bleomycin and the APE1/NPM1 inhibitors, the indicated amount of bleomycin was mixed with 10 μM inhibitor and treatment was carried out for 1 h, medium was replaced and cells were allowed to form colonies in fresh medium.

For cellular growth rate measurements, 2×10^4 cells were seeded in 24-well plates, treated 24 h later and counted at the indicated time points using a coulter counter (Beckman Coulter).

DNA Damage Accumulation Measurements

Genomic DNA was isolated from 1×10^6 HeLa cells by using the Get *pure*DNA Kit (Dojindo, Rockville, MD) and AP-sites content was measured by using the DNA Damage Quantification Kit (Dojindo) as per manufacturer's indications. Briefly, 1 μg of genomic DNA was labeled with a biotinylated aldehyde reactive robe (ARP) for 1 h at 37°C, and ARP-DNA was purified following the manufacturer's instructions. The amount of labeled ARP–DNA was then quantified through a colorimetric reaction and eventually measured using a calibration curve provided with the kit.

Alkali comet assay was carried out essentially as described in ref. [30]. Briefly, HeLa cells were exposed to the indicated concentrations of the APE1/NPM1 inhibitors for 16 h and washed twice with ice-cold PBS. Approximately 3×10^3 cells were embedded on slides in 0.5% (w/v) low melting point agarose (Cambrex Corporate, East Rutherford, NJ) in PBS. The slides were placed in cold lysis solution (2.5 M NaCL, 100 mM EDTA, and 10 mM Tris pH 10.0, 1% (v/v) Triton X-100) for 1 h at 4°C, and washed in cold 0.4 M Tris (pH 7.4). Next, the slides were incubated in alkali solution (300 mM NaOH, 1 mM EDTA, final pH 13.0) for 30 min at 4°C and then electrophoresed horizontally for 30 min at 25 V, 350 mA at 4°C. Slides were briefly neutralized with 0.4 M Tris (pH 7.4), stained with ethidium bromide (2 μg/ml) and viewed on a Zeiss Axiovert 200 M fluorescent microscope (Zeiss, Thornwood, NY). The analysis of the comet tail was carried out using the Comet Assay IV software (Perceptive Instruments, Suffolk, UK) to determine the Olive tail moment. At least 100 cells per experimental condition were scored.

rRNA Maturation Kinetics

rRNA processing was monitored as described in ref. [31] with minor modifications. In brief, 5×10^5 HeLa cells were treated with the indicated inhibitors. For metabolic labeling cells were trypsinized and immediately phosphate-depleted under rocking at 37°C for 1 h in presence of unchanged drug concentrations, by incubation in phosphate-free DMEM supplemented with 10% (v/v) dialyzed FBS (Invitrogen). Medium was then replaced with phosphate-free DMEM supplemented with 10% (v/v) dialyzed FBS containing 15 μCi/ml [^{32}P]orthophosphate (Perkin Elmer, Milan, Italy) and cells were labeled for 1 h. Medium was again replaced with normal DMEM supplemented with 10% (v/v) FBS in presence of unchanged drug concentrations and total RNA was isolated after 2 h using the Trizol® Reagent (Invitrogen). RNA was separated on an agarose-formaldehyde gel loading the same amount of radioactivity per lane. Gels were vacuum-dried and subjected to autoradiography.

AP-Site Incision Assays

APE1 endonuclease activity was monitored using purified recombinant APE1. Enzymatic reactions were carried out in a final volume of 10 μl using 2.3 fmol of protein in a buffer containing 50 mM HEPES pH 7.5, 50 mM KCl, 10 mM $MgCL_2$, BSA 1 μg/μl and 1 mM DTT; samples were pre-incubated for 15 min at 37°C with 230 pmol of the selected inhibitors. Reactions were started by adding 100 nM of double stranded abasic DNA substrate (obtained by annealing a DY-782-labeled oligonucleotide 5′-CTTGGAACTG-GATGTCGGCACFAGCGGATACAGGAGCA-3′ (Dyomics), where F indicates a tetrahydrofuran residue, with the complementary sequence 5′-TGCTCCTGTA-TCCGCTGTGCCGACATCCAGTTCCAAG-3′) and incubated at 37°C for the indicated time points.

Reactions were halted by addition of formamide buffer (96% formamide, 10 mM EDTA and gel Loading Buffer 6× (Fermentas)), separated onto a 20% (w/v) denaturing polyacrylamide gel and analyzed on an Odyssey CLx scanner (Li-Cor Biosciences). The percentage of substrate converted to product was determined using the ImageStudio software (Li-Cor Biosciences).

Statistical Analyses

Statistical analyses were performed by using the Student's *t* test. $P < 0.05$ was considered as statistically significant.

RESULTS

An AlphaScreen® - Based High-Throughput Screening Assay for the Identification of Low Molecular Weight APE1/NPM1 Interaction Disruptors

Screening for low molecular weight compounds that target a protein–protein interaction interface is a challenging task. In order to find molecules able to impair the APE1/NPM1 association, we exploited the AlphaScreen® technology using recombinant purified full length GST-tagged APE1 and $His_{(6)}$-tagged NPM1. The AlphaScreen® assay relies on a proximity-based reporting system used to measure the binding between two cognate partners. Laser excitation at 680 nm of a colloidal-size donor bead releases a flow of singlet oxygen (Figure 1A). Acceptor beads in close proximity (<200 nm) utilize this singlet oxygen to generate a chemiluminescence signal emitting in the 520–620 nm range. Here, we have configured an AlphaScreen® assay by using glutathione-coated donor beads and Ni^{2+} chelate-coated acceptor beads to measure binding of GST-APE1 to $His_{(6)}$-NPM1. To search for compounds with the ability to disrupt the APE1/NPM1 interaction, we screened a pilot set of libraries composed of 12 700 small molecules at a seven-concentration dilution series (3.7 nM–57 μM). An excellent Z′ score (>0.65) was maintained throughout the screen (Figure 1B). Compounds that

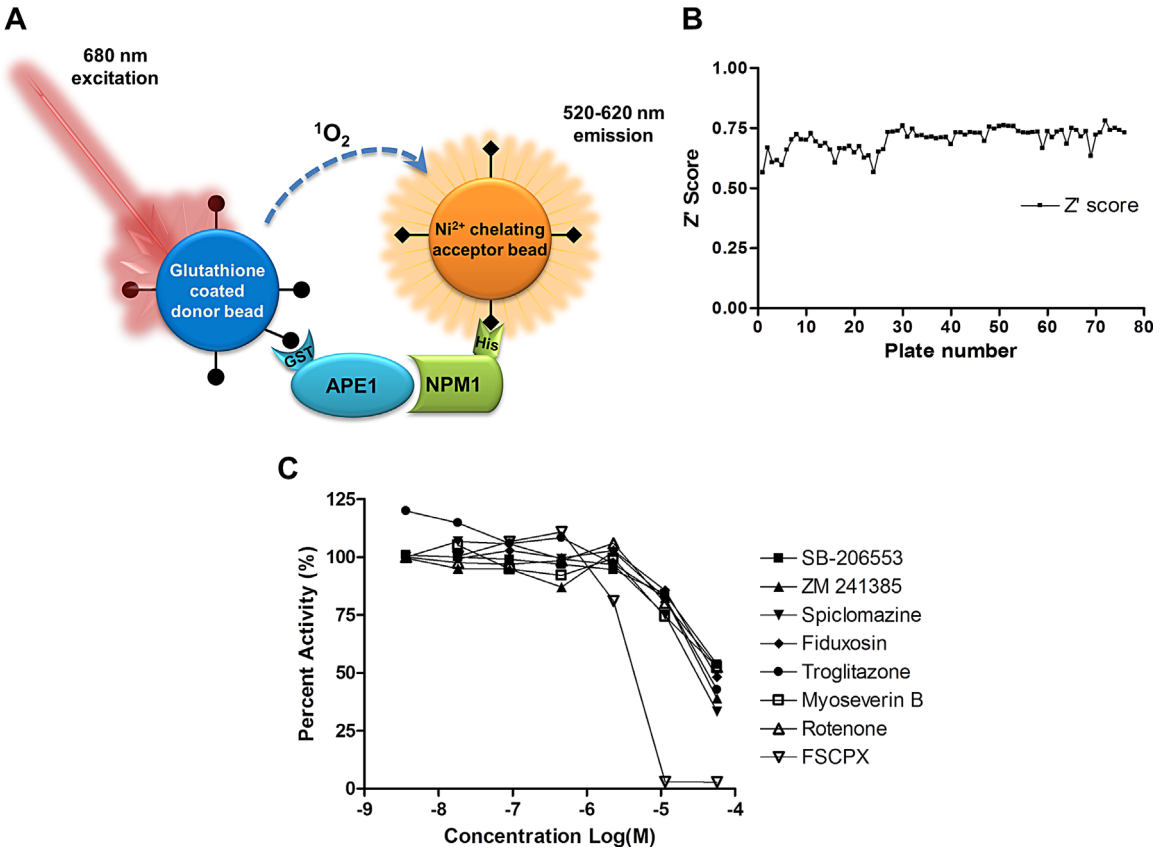


Figure 1. Development of an HTS assay to individuate APE1/NPM1 interaction inhibitors. (A) Schematic representation of the HTS assay principle. The interaction between full length GST-APE1 and $His_{(6)}$-NPM1 is monitored in solution upon excitation at 680 nm. The glutathione-coated donor beads release singlet oxygen, which excites only proximal (≤200 nm) Ni^{2+}-chelating acceptor beads. The presence of APE1/NPM1 interaction is revealed by luminescence emission at 520–620 nm. (B) Graphic summary reporting the Z' screening score; a stable value above 0.65 was maintained throughout the screening. (C) Representative curves observed from eight screening hits selected during downstream analyses. The graph reports the concentration-dependent loss of APE1/NPM1 interaction signal for the indicated compounds, relative to the vehicle-treated samples. Structures and additional data associated with the best characterized hits are presented in Figure 5.

showed inhibitory activity in the primary screen were rescreened for confirmation. To eliminate false positives, we used a counterscreen assay to measure the binding AlphaScreen® beads to a GST-$His_{(6)}$ conjugate, which served as a recognition moiety for both donor and acceptor beads outside the context of the APE1/NPM1 interaction. With this approach, we confirmed 58 compounds as true hits (chosen based on availability, Supplementary Table 1), which were used in further downstream analyses. Representative dose-response curves for a subset of positive compounds are reported in Figure 1C.

Secondary Validation Assays for the Selection of Molecules Able to Impair the APE1/NPM1 Interaction in Living Cells

The positive hits from the primary screening analyses were subjected to a panel of orthogonal cell-based secondary validation assays in order to focus on those molecules able to impair the APE1/NPM1 association in living cells. HeLa cells are a well-characterized cell model that has been widely used by our and other laboratories to study APE1 biology [32–35] and to screen for APE1 inhibitors [36,37]. Thus, we used them as a general model to probe the activity of the putative APE1/NPM1 inhibitors. All 58 of the initial positive compounds was preliminarily tested over a wide range of doses (0.1–100 μM) and time points (4–24 h) assessing cell viability through the MTS assay. These initial experiments allowed us to estimate the range of solubility in the cell medium (evaluating particle deposition through a phase contrast microscope) and the cytotoxicity for each compound (not shown).

As the interaction with NPM1 is known to modulate the subcellular localization of APE1 [21,22], we used an immuno-fluorescence-based approach to detect any change in subcellular distribution of APE1 upon treatment with the molecules. Timing and dosage of the treatments were selected on the basis of the individual toxicity and solubility of every compound. In order to reduce the likelihood of confounding effects, for each of the 58 putative inhibitors, we tested at least two conditions (time and/or dosage) at which cell viability was affected by less than 50%. Treatment with the molecules affected the APE1 subcellular localization in different ways (Supplementary Table 1): a number of compounds (e.g., SB 206553, spiclomazine, etc.) increased the cytoplasmic localization of APE1, while others (e.g., ZM 241385, troglitazone, fiduxosin) led to a reduction of the APE1 nucleolar accumulation (Figure 2A).

Only compounds affecting APE1 staining (17 out of 58—Supplementary Table 1) were further screened through a proximity ligation assay (PLA) [22,28,29], which was designed to measure the extent of the residual APE1/NPM1 association upon cell treatment. As the AlphaScreen® was carried out using a recombinant GST-tagged form of APE1, we sought to exclude any possible artifact arising from the presence of the N-terminal tag by exploiting a HeLa cell line stably expressing a C-terminally FLAG-tagged version of APE1 [21]. The presence of the tag, in this case, was necessary to improve the quality of the PLA signal, and was already shown not to affect the interaction with NPM1 [22]. PLA was carried out by fixing cells immediately after treatment with the putative inhibitors and revealing the molecular association between APE1 and NPM1 with an anti-FLAG/anti-NPM1 pair of antibodies (Figure 2B). Quantification of the PLA signal allowed us to determine whether a particular compound was able to decrease the extent of the APE1/NPM1 interaction below that of vehicle-treated cells. This approach allowed us to narrow the number of putative inhibitors to eight molecules effectively able to interfere with the APE1/NPM1 interaction in cells (Figure 2C—top panel). As anticipated by the immuno-fluorescence preliminary screening, some of the compounds that caused relocalization of APE1 (e.g., fiduxosin, spiclomazine) led to an accumulation of the interaction signal from the nucleoplasm to the cytoplasm (Figure 2C—bottom panel). Treatment with other compounds, while leading to APE1 relocalization to the cytoplasm, did not produce any increase in the APE1/NPM1 interaction in this cellular compartment (e.g., SB 206553). In summary, our approach, exploiting a panel of secondary cell-based screens, allowed us to validate a set of eight low molecular weight compounds (i.e., FSCPX, troglitazone, SB 206553, ZM 241385, rotenone, spiclomazine, fiduxosin, and myoseverin B) able to effectively displace the APE1/NPM1 interaction upon cell treatment. Rotenone, a respiratory chain poison and myoseverin B, known to bind microtubules [38], were excluded from additional analyses, because of their previously noted off-target effects.

To better characterize the six remaining hits, we analyzed their ability to interfere with the APE1 redox function. We exploited the JHH6 hepatocarcinoma cell line, which has already been shown to activate NF-κB in an APE1-mediated and redox-dependent manner after a challenge with TNF-α [27]. JHH6 cells were transfected with a reporter plasmid bearing an interleukin 8 (IL-8) promoter with NF-κB consensus sequences, and treated with the APE1/NPM1 inhibitors prior to TNF-α addition (Figure 3A). Most of the tested molecules had no or little effect on the basal IL-8 promoter transcription; the effect of the APE1/NPM1 inhibitors on the redox-dependent promoter activation ranged from strong (e.g., troglitazone), mild (e.g., spiclomazine) to null (e.g., SB 206553). As expected [27], cell pre-treatment with the APE1 redox inhibitor E3330, strongly impaired the TNF-α-induced NF-κB

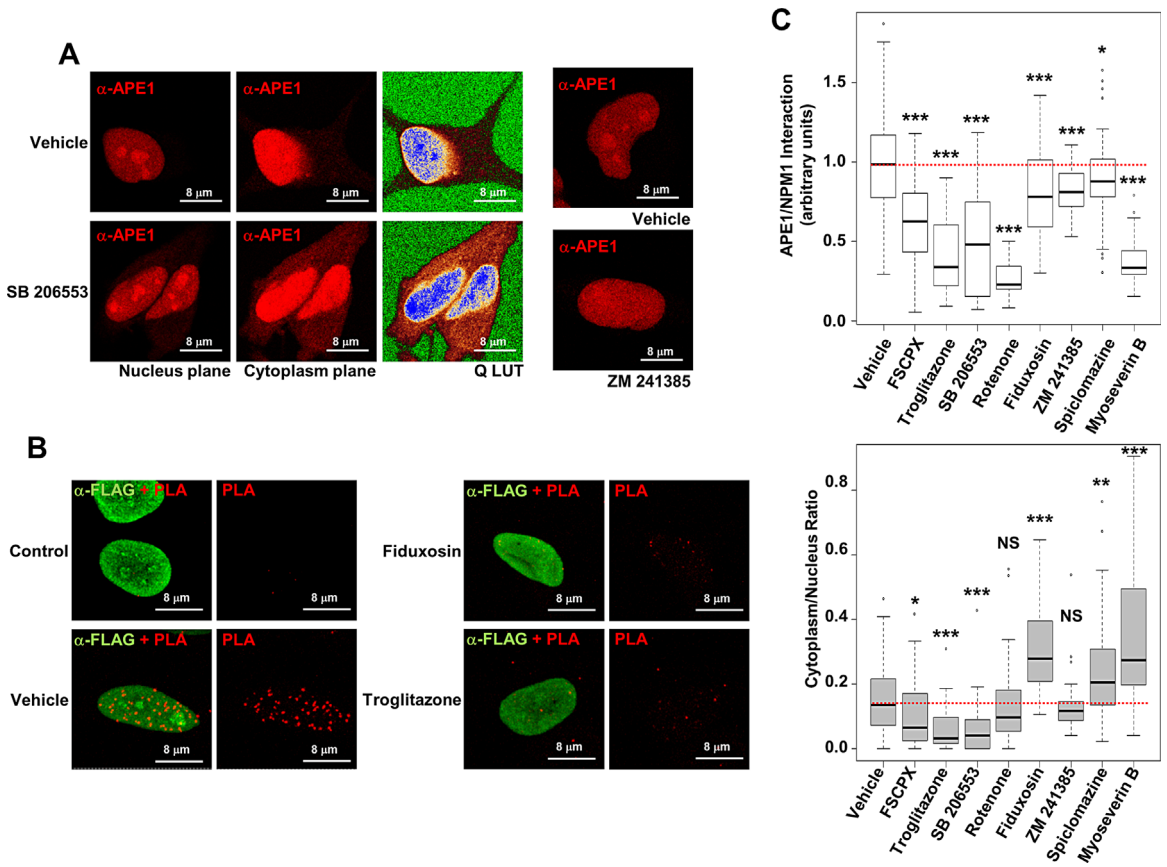


Figure 2. Secondary orthogonal assays to validate the hits from the primary screening. (A) Representative immuno-fluorescence analysis on HeLa cells treated with positive hits from the primary screening. The subcellular localization of APE1 was assessed through immuno-staining with an anti-APE1 antibody (red). Note the absence of nucleolar accumulation of APE1 upon stimulation with ZM 241385. Q LUT (quantitative color look-up table) rendering highlights the increased cytoplasmic localization after SB 206553 treatment. (B) PLA-based secondary validation of the APE1/NPM1 inhibitors. Representative PLA experiment performed on fixed HeLa cells upon treatment with fiduxosin (10 μM, 16 h) or troglitazone (10 μM, 8 h). APE1 expression is detected by using an anti-FLAG antibody (green), while PLA signal is visible as red dots. Control reaction is carried out without the anti-NPM1 antibody and shows no or little PLA signal. (C) The extent of APE1/NPM1 association upon treatment with a subset of inhibitors was measured through PLA, and the effect of the inhibitors on such interaction is reported as a boxplot graph (top panel). The average number of interaction spots scored in vehicle-treated cells was used as reference and arbitrarily set to 1; the relative extent of APE1/NPM1 association upon inhibition of the interaction was calculated by scoring the average of PLA signals in the inhibitor-treated cells. Cell challenge with the indicated molecules determines a statistically significant reduction in the extent of cellular APE1/NPM1 interaction. Boxplot representation reporting the median cytoplasm-to-nucleus ratios of the amount of PLA interaction spots scored in HeLa cells upon treatment with the indicated APE1/NPM1 inhibitors (bottom panel). Black bars in the graphs report the median for each distribution. A red line indicates the median of the vehicle-treated cells, for easier identification of differences. The amount and the localization of the APE1/NPM1 interaction signals were assessed as described in "Materials and Methods." $N \geq 35$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS: not statistically significant.

activation. Notably, the APE1/NPM1 inhibitor troglitazone had an effect similar to E3330, even at a tenfold lower concentration.

Selected Compounds Sensitize Tumor Cells to Genotoxins and Display Anti-Proliferative Activity as Single Agent

We next focused on the anti-tumor properties of the six selected APE1/NPM1 inhibitors. We investigated their ability to sensitize HeLa cells to different genotoxins. Cell viability was measured through the MTS assay upon pre-treatment with the inhibitors, followed by challenge with methyl-methanesulphonate (MMS), which induces DNA alkylation that is repaired by the BER pathway [39]. Pre-treatment of cells with SB 206553 or spiclomazine resulted in a synergistic cytotoxicity when combined with MMS (Figures 3B and 5). Likewise, through both cell counting and colony formation experiments, a sensitization effect was observed when combining fiduxosin, spiclomazine, or SB 206553 with the radiomimetic drug bleomycin, which is known to induce DNA strand breaks, along with a subset of oxidative DNA lesions that are repaired with the intervention of APE1 [40] (Figures 3C, D and 5). Neither FSCPX nor ZM 241386 had a statistically significant effect on cell viability in combination with any genotoxic drug (not shown), and were therefore excluded from further characterization.

In addition to the enhancement of the cytotoxic effect of therapeutically relevant DNA damaging drugs, a desirable feature for an adjuvant to conventional anti-tumor therapy is an anti-proliferative

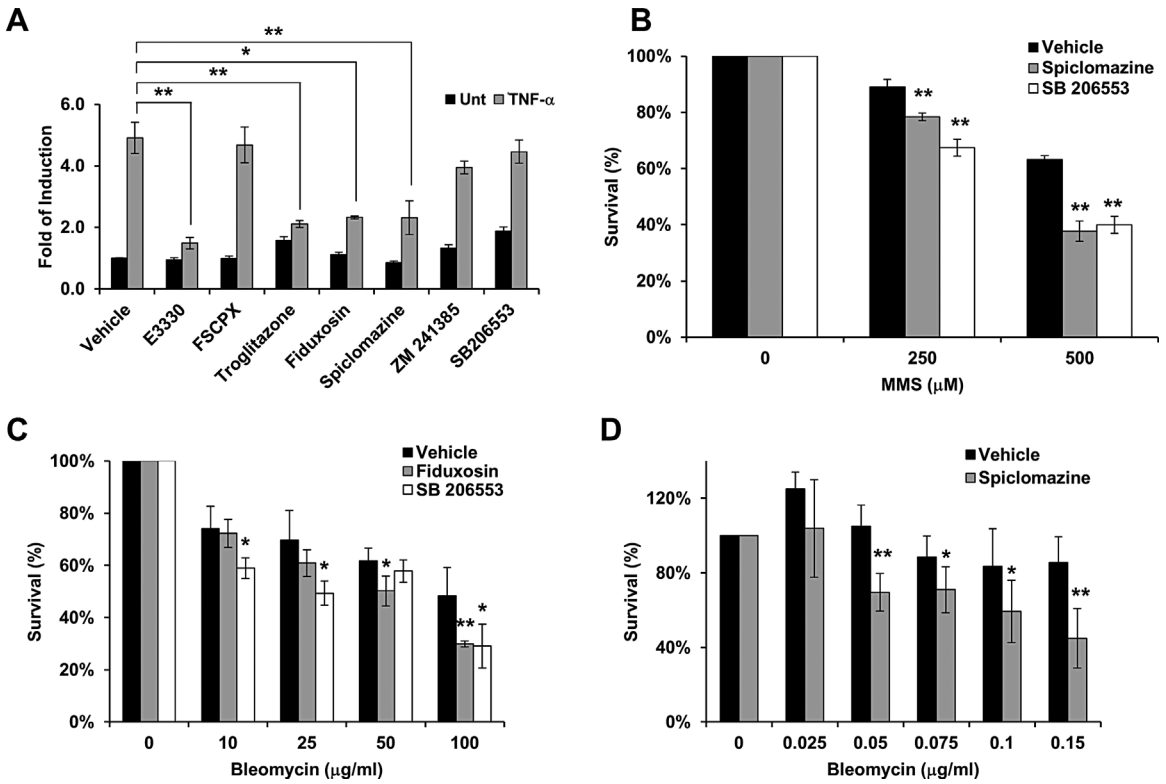


Figure 3. Inhibition of the APE1/NPM1 interaction has differential impact on the APE1 redox activity and sensitizes HeLa cells to genotoxic treatment. (A) The APE1/NPM1 inhibitors differently affect the redox function of APE1. JHH6 cells were transfected with a luciferase reporter bearing the IL-8 promoter with NF-κB consensus sequences [27] and then pre-treated with the indicated APE1/NPM1 inhibitors. After 5 h cells were challenged with TNF-α and luciferase activity was measured to assess the APE1-mediated NF-κB activation. While the inhibitors activity on APE1 basal redox activity was negligible, they showed differential inhibitory capacity over TNF-α-mediated NF-κB activation. Results reported are the mean ± SD of at least three independent experiments, $*P < 0.05$, $**P < 0.01$. E3330 was used as positive control for inhibition of APE1. (B) SB 206553 and spiclomazine sensitize HeLa cells to MMS. HeLa cells were pre-treated with 10 μM SB 206553 or spiclomazine for 8 h and then co-treated for further 8 h with unchanged inhibitor concentration and MMS. The effect of SB 206553 and spiclomazine as single agent was, respectively, 1.08 ± 0.08% and 0.99 ± 0.04% relative to the vehicle. (C) Bleomycin cytotoxicity is increased in presence of either SB 206553 or fiduxosin. HeLa cells were co-incubated with increasing amounts of bleomycin in presence of 10 μM of the indicated APE1/NPM1 inhibitor for 1 h. Cell viability was monitored 48 h later through cell counting. The effect of SB 206553 and fiduxosin as single agent was, respectively, 1.22 ± 0.02% and 0.86 ± 0.06% relative to the vehicle. (D) Combination with spiclomazine increases the potency of bleomycin. HeLa cells were co-incubated with increasing amounts of bleomycin in presence of 10 μM spiclomazine for 1 h. Cell viability was monitored through colony formation assays, showing synergic behavior of bleomycin and spiclomazine. The cell killing effect of spiclomazine as single agent was 0.90 ± 0.08% relative to the vehicle. Data reported are the mean ± SD of at least three independent experiments, $*P < 0.05$, $**P < 0.01$.

activity. We tested whether the three inhibitors that promoted sensitization to MMS or bleomycin had any effect on cellular proliferation as a single agent. Remarkably, treatment of HeLa cells with either fiduxosin or spiclomazine, but not with SB 206553, resulted in a dose-dependent reduction of the cellular growth rate. This effect was visible at sub-lethal dosage (5–20 μM), both upon acute (24–72 h) or chronic (10 days) treatment (Figures 4A, B and 5).

Characterization of the Molecular Target of the Top Hit Compounds

In order to understand whether the molecular target of the inhibitors is APE1 or NPM1, we analyzed their binding properties using surface plasmon resonance (SPR). Despite the absence of any synergistic effect in combination with genotoxins (not shown), troglitazone was also characterized in these assays, given its powerful inhibitory effect on the APE1 redox activity (Figure 3A). SPR experiments showed that the four most promising small molecules preferentially bind immobilized recombinant APE1, rather than NPM1 (Figure 6A). Note that fiduxosin and spiclomazine could not be used at higher concentration due to their limited solubility in aqueous buffer. We then compared the binding affinity of SB 206553 and troglitazone to mutant forms of APE1. The interaction with both small molecules was severely impaired by the loss of the APE1 unstructured N-terminal extension, which is responsible for the interaction with NPM1 [21] (Figure 6B, compare APE1 WT and the APE1 NΔ33 truncation mutant). Comparison of the APE1 WT and the APE1 KA form, which preserves the N-terminal extension but loses its ability to interact with NPM1 [21], revealed no difference in the binding capacity of the inhibitors (Figure 6B).

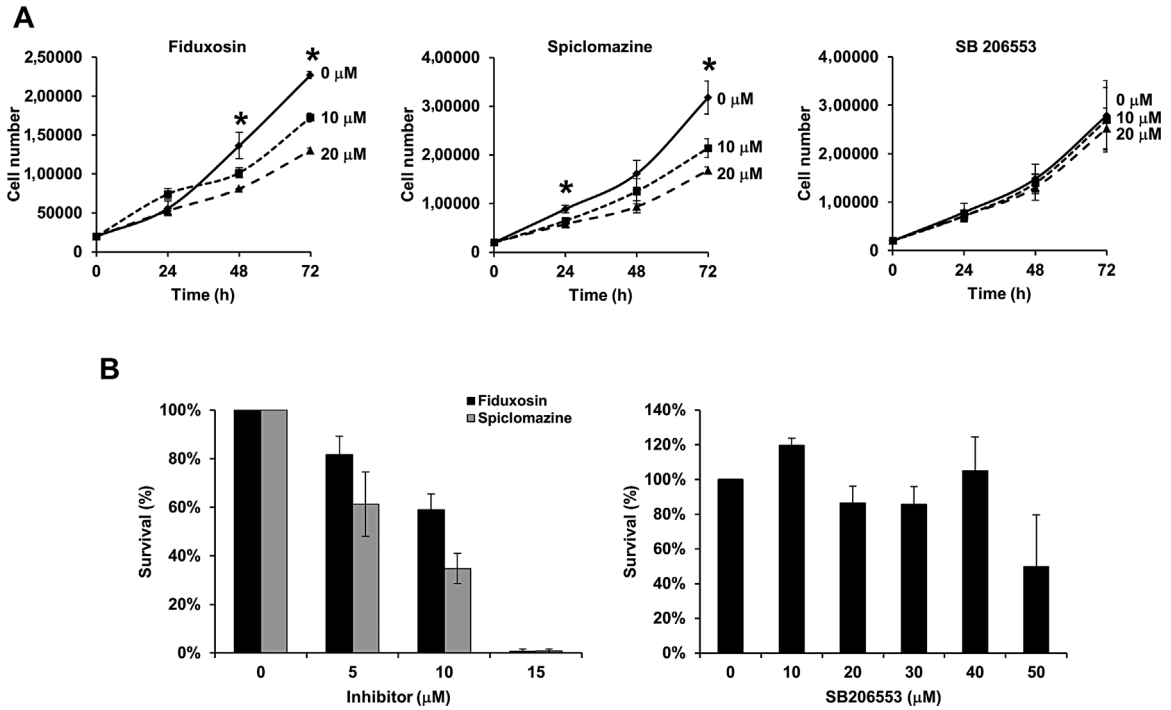


Figure 4. Fiduxosin and spiclomazine, but not SB 206553, impair cell growth as single agents. (A) HeLa cells were grown in presence of the indicated APE1/NPM1 inhibitor (10 or 20 μM) and cell proliferation was assessed after 24, 48 and 72 h through cell counting. Acute treatment with fiduxosin or spiclomazine, but not with SB 206553, affects the cellular proliferation rate in a dose-dependent fashion. (B) HeLa cells were chronically stimulated with increasing amounts of the indicated inhibitor during colonies growth. The percentage of surviving colonies relative to vehicle-treated cells is reported. Inhibition of the APE1/NPM1 interaction using fiduxosin or spiclomazine, but not SB 206553, results in a dose-dependent impairment of colony formation capacity. Data reported are the mean ± SD of at least three independent experiments, $*P < 0.05$.

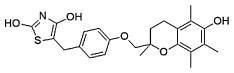
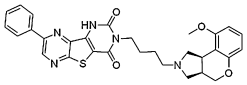
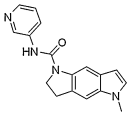
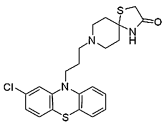
Compound	Structure	Redox inhibition	AP-site incision inhibition	Sensitization to genotoxins		Anti-proliferative activity
				MMS	Bleomycin	
Troglitazone		++	+	-	NA	NA
Fiduxosin		+	++	-	+	+
SB 206553		-	+	+	+	-
Spiclomazine		+	++	+	+	+

Figure 5. Sensitizing and anti-proliferative capacity of the APE1/NPM1 inhibitors. The figure schematically summarizes the properties for each top-hit inhibitor. For every compound analyzed the "+" symbol indicates the presence of sensitization in combination with the indicated genotoxin. "++" and "+" indicate the strength of the phenotype on the APE1 redox and AP-endonuclease functions, as strong or mild, respectively. The anti-proliferative activity of each molecule as single-agent is also reported, along with the chemical structure and the commercial name of the compound. NA: not available, "−": no effect.

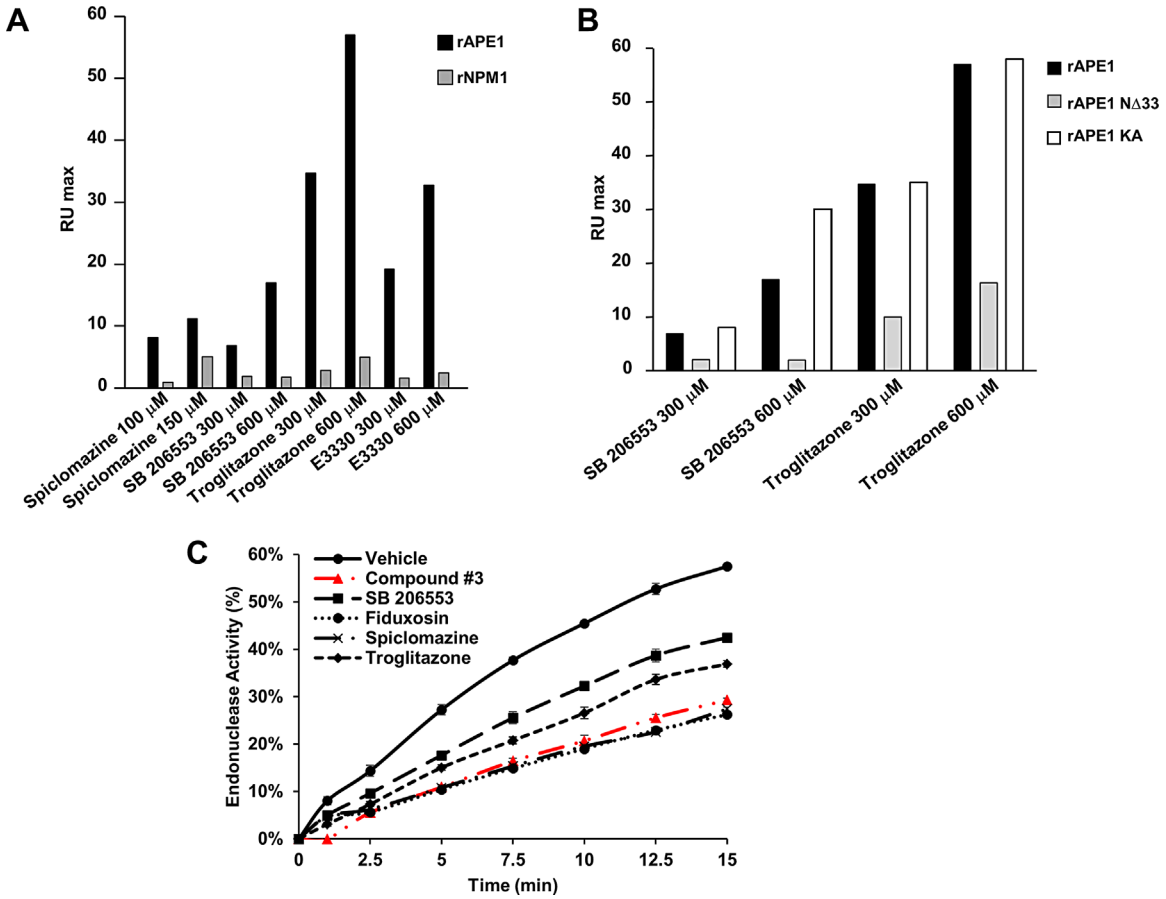


Figure 6. The APE1/NPM1 inhibitors show preferential binding to APE1. (A) Histogram representation of the SPR analysis on the small molecules–protein interaction studies. The graph reports the RU_{max} for each binding experiment using the indicated concentration of analyte on immobilized recombinant APE1 or NPM1; Note the lower response for NPM1. E3330 was used as positive control for interaction with APE1. (B) The indicated recombinant APE1 forms were compared for the interaction with SB 206553 or troglitazone The histogram reports the RU_{max} for each binding experiment using the indicated concentration of analyte; signal was normalized to the molecular weight of each small molecule. (C) The APE1/NPM1 inhibitors negatively impact on the AP-site incision activity of APE1. APE1 endonuclease activity was measured in vitro as indicated in the "Materials and Methods" section in presence of 100-fold molar excess of the indicated molecules. APE1 activity is reported as percentage of substrate converted to product as a function of time. The APE1 inhibitor compound #3 [37] was used as positive control; the APE1 AP-site incision profile in presence of this inhibitor is highlighted in red. Data are expressed as mean ± SD of three technical replicates from two independent assays.

Taken together, these data suggest that APE1, rather than NPM1, is the preferred binding partner for some of the molecules active in this assay.

The above observations were further corroborated by in vitro assays assessing the endonuclease activity of APE1. These experiments showed that the inhibitors, at least slightly, inhibited the catalytic incision activity of purified recombinant APE1, with some molecules (i.e., spiclomazine and fiduxosin) having an effect comparable to that of the known APE1 inhibitor compound #3 [37] (Figure 6C and Supplementary Fig. S1).

The APE1/NPM1 Inhibitors Do Not Impair Ribosome Biogenesis and Only Fiduxosin Leads to Accumulation of AP-Sites

It is conceivable that the effects of the APE1/NPM1 inhibitors are mediated by the downregulation of one or both proteins. Notably, none of the three molecules effective in the combination studies (i.e., fiduxosin, spiclomazine, and SB 206553) led to a significant reduction of either APE1 or NPM1 protein amounts (Figure 7). Thus, neither the observed APE1/NPM1 interaction impairment, nor the measured effect on cell

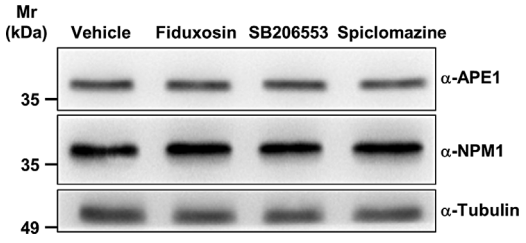


Figure 7. Inhibition of the APE1/NPM1 interaction does not affect the expression levels of APE1 or NPM1.Representative Western blotting showing the APE1 and NPM1 expression levels in HeLa whole cell extracts. After treatment with the indicated inhibitors (10 μM for 16 h) no significant variation of the protein expression pattern is observed.

growth or sensitivity to DNA damage, can be ascribed to variations in the expression levels of these proteins.

Focusing on fiduxosin or spiclomazine, immunofluorescence analyses revealed that NPM1 maintains its nucleolar residence over a 72 h treatment with either compound (Figure 8A). Over the same time course, APE1 did not change its nucleolar localization when cells were treated with spiclomazine. On the other hand, in cells treated with fiduxosin, APE1 was depleted from nucleoli during the first 48 h (Figure 8B), as already observed during the immuno-fluorescence based secondary screening (Supplementary Table 1). These observations suggest that the cytostatic effect of these two APE1/NPM1 inhibitors is not driven by the disruption of nucleolar integrity, as NPM1 localization was unaffected [31]. Fiduxosin, in this case, represented a notable exception, showing intact nucleoli, but relocalization of APE1. In accordance with the absence of nucleolar disruption, the ribosome processing kinetics in HeLa cells treated with either fiduxosin or spiclomazine did not show any obvious alteration (Figure 9A), indicating that other mechanisms might account for the impairment in cell proliferation.

To gain further insight into the mechanism of action of the selected APE1/NPM1 inhibitors we assessed any inhibitor-dependent accumulation of DNA damage through measurement of AP-sites and the use of the alkaline comet assay. Our data indicate that treatment of HeLa cells with fiduxosin induced a small, albeit significant, increase in the genomic AP-site content (Figure 9B); spiclomazine or SB 206553, conversely, did not induce any major accumulation of DNA damage (Figure 9B and C).

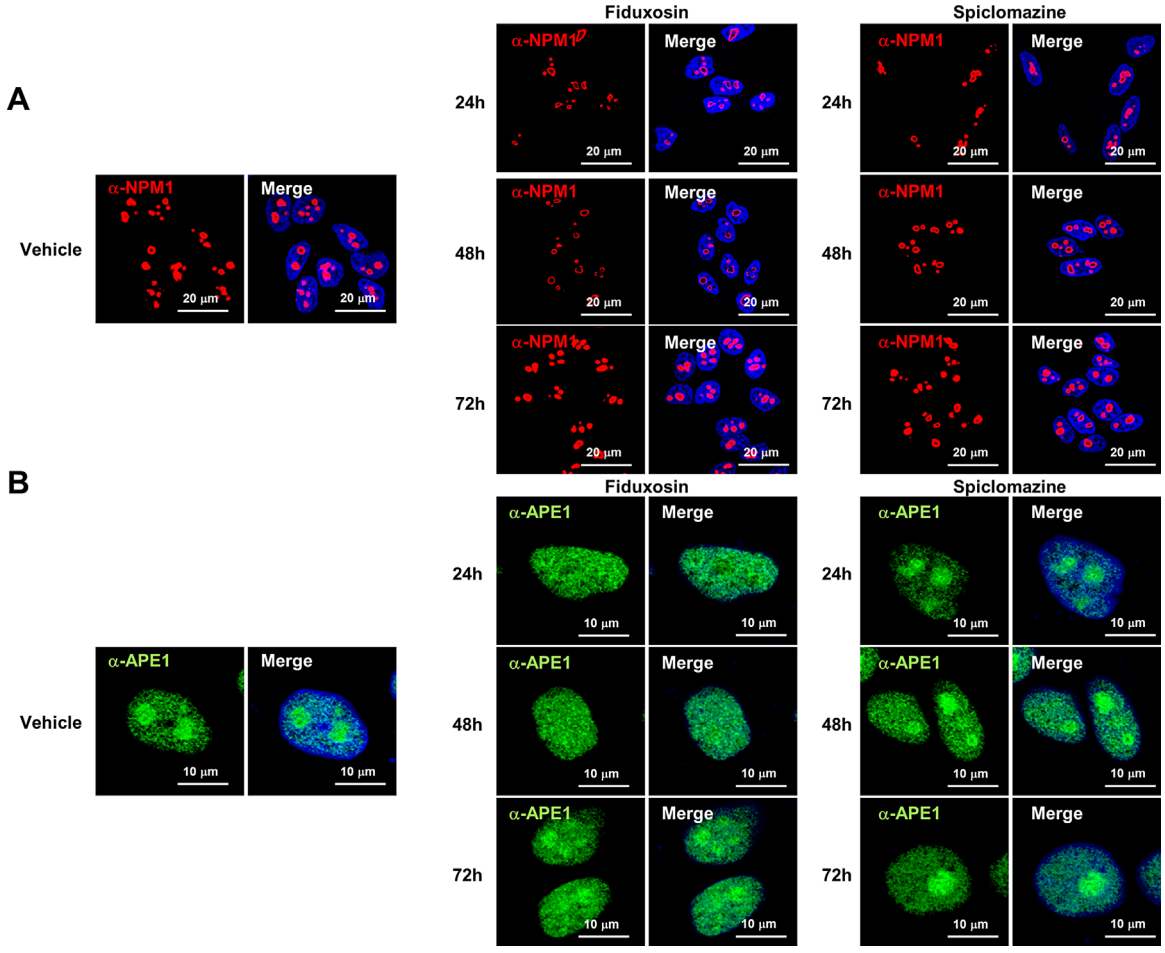


Figure 8. NPM1 localization is not affected by prolonged treatment with fiduxosin or spiclomazine; whereas APE1 nucleolar accumulation is affected by fiduxosin only. (A) Representative immuno-fluorescence analysis on HeLa cells treated with either fiduxosin or spiclomazine (20 μM for the indicated time points). NPM1 staining (red) shows the constant nucleolar accumulation of the protein throughout the treatment. Merged panels show the superimposition of NPM1 and TO-PRO-3 staining (blue). (B) Representative micrographs showing a typical immuno-fluorescence analysis on HeLa cells treated with either fiduxosin or spiclomazine (20 μM for the indicated time points). APE1 staining (green) shows a decreased nucleolar accumulation of the protein in presence of fiduxosin, but not upon treatment with spiclomazine. Merged panels show the superimposition of APE1 and TO-PRO-3 staining (blue).

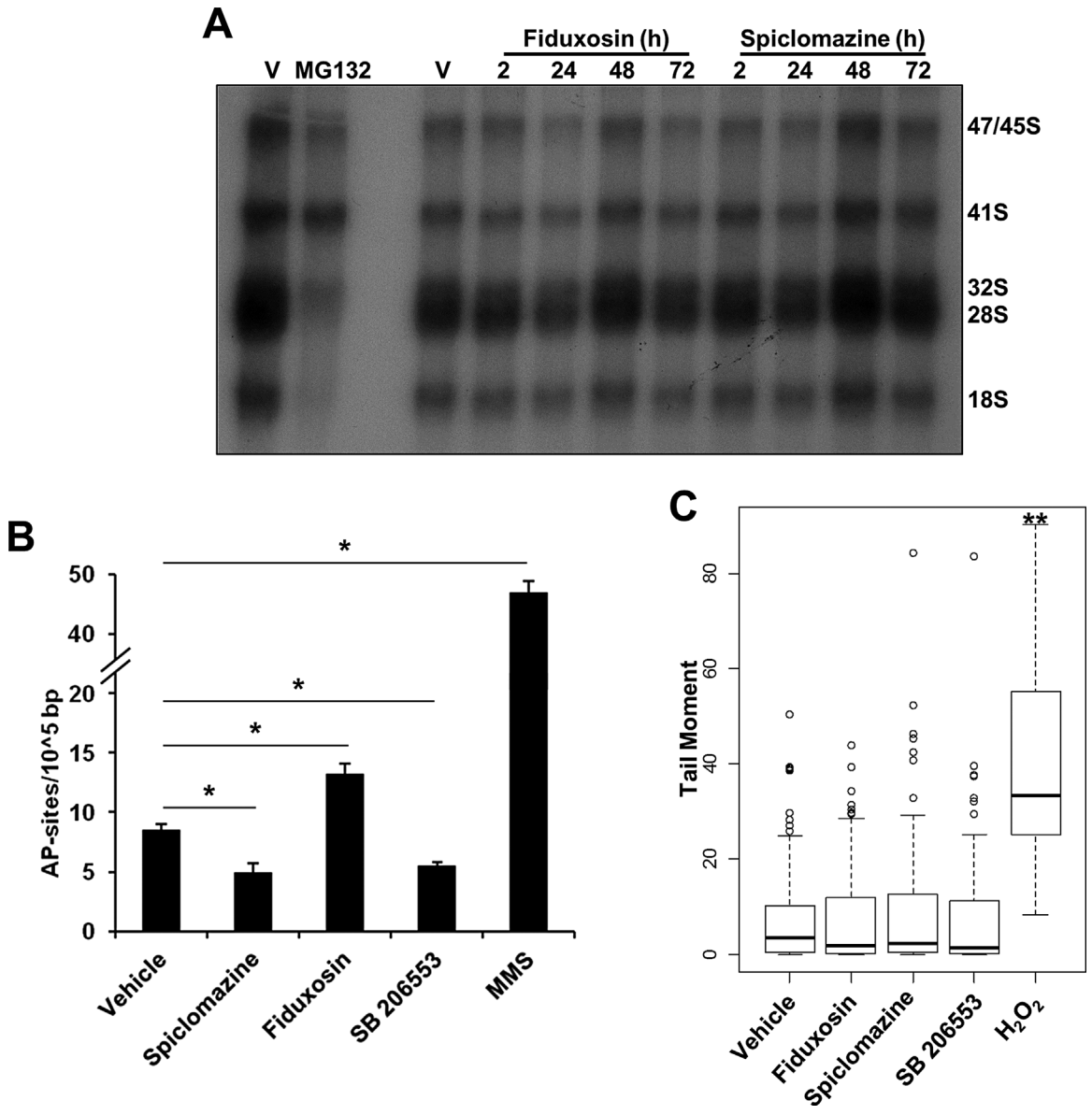


Figure 9. Inhibition of the APE1/NPM1 interaction does not affect rRNA processing or DNA integrity. (A) rRNA maturation kinetics were monitored in HeLa cells as described in "Materials and Methods" after treatment with either spiclomazine or fiduxosin (20 μM) for the indicated time. The proteasome inhibitor MG132 (50 μM, 2 h) was used as positive control [31]. Cell treatment under these experimental conditions does not lead to any obvious rRNA processing impairment. V: vehicle. (B) Genomic AP-site content quantification on HeLa cells upon treatment with the APE1/NPM1 inhibitors does not reveal any major damage accumulation. HeLa cells were treated with fiduxosin or spiclomazine (10 μM, 48 h), SB 206553 (20 μM, 16 h), or MMS (250 μM, 16 h) as positive control. AP-sites amount was calculated as described in "Materials and Methods." Data are expressed as mean ± SD of three replicate measurements, $*P < 0.01$. (C) Comet assay performed on HeLa cells shows the lack of accumulation of alkali-sensitive sites on genomic DNA after treatment with the APE1/NPM1 inhibitors (16 h). HeLa cells were exposed to fiduxosin, spiclomazine (30 μM), SB 206553 (100 μM), or H_2O_2 (200 μM, 10 min) as positive control, and tail moment was measured as described. The boxplot reports the median tail moment in at least 100 cells per condition. $** P < 0.001$.

The Cytotoxic Effect of the APE1/NPM1 Is Common to Different Tumor Cell Lines

In order to extend our findings to other cell lines, we estimated the IC_{50} of fiduxosin, spiclomazine and SB 206553 on a panel of tumor cell lines representing different cancer histotypes, such as hepatic (Huh7, JHH6), breast (HCC70, MCF7), glial (SF767), and ovarian (Ovcar5, TOV-112D). As shown in Table 1 and in Figure 10, the IC_{50} of the APE1/NPM1 inhibitors was similar among the samples, with few cell line-specific exceptions. Notably, HeLa cells were quite resistant to the cytotoxicity induced by the APE1/NPM1 inhibitors, having very high IC_{50} values. Sensitization assays were also carried out on TOV-112D. Here, the sensitization effect of fiduxosin and spiclomazine was reproduced in the TOV-112D

Table 1. IC_{50} Estimates for Spiclomazine, Fiduxosin and SB 206553 on Different Tumor Cell Lines

Compound	HeLa	Huh7	HCC70	JHH6	SF767	MCF7	Ovcar5	TOV-112D
Fiduxosin	NA	39.8	43.5*	31.3	43.3*	NA	52.7*	45.6*
Spiclomazine	NA	38.0	46.3*	48.8*	NA	NA	NA	40.2
SB 206553	109.4*	NA	56.0	84.2	55.8	77.5	NA	NA

The indicated cell lines were exposed to the APE1/NPM1 inhibitors for 48 h and cell viability was assessed using the MTS assay. IC_{50} values are expressed in μM and were calculated using the GraphPad Prism v6.0 software. Starred values (*) are slightly above the experimental curve and were therefore extrapolated using the same software. "NA" indicates an IC_{50} far above the tested range.

ovarian cancer cell line with bleomycin (Figure 11A), but not with MMS. In addition, viability assays comparing the AML cell lines OCI/AML-2 (expressing wild-type NPM1) and OCI/AML-3(expressing the NPM1c mutant and having an impaired APE1/NPM1 interaction [22]), showed a sensitizing effect for the SB 206553-bleomycin combination only in the cell line expressing wild-type NPM1 (Figure 11B).

Altogether, these data suggest that the cytotoxic and the sensitizing effects of spiclomazine, SB 206553 and fiduxosin are not restricted to HeLa cells. Moreover, these data further suggest that a functional APE1/NPM1 interaction is likely needed for the SB 206553 sensitization effect to occur in AML cell lines.

DISCUSSION

The study presented herein represents the first attempt to target the APE1 protein in tumor cells through the disruption of its interactome. Exploiting

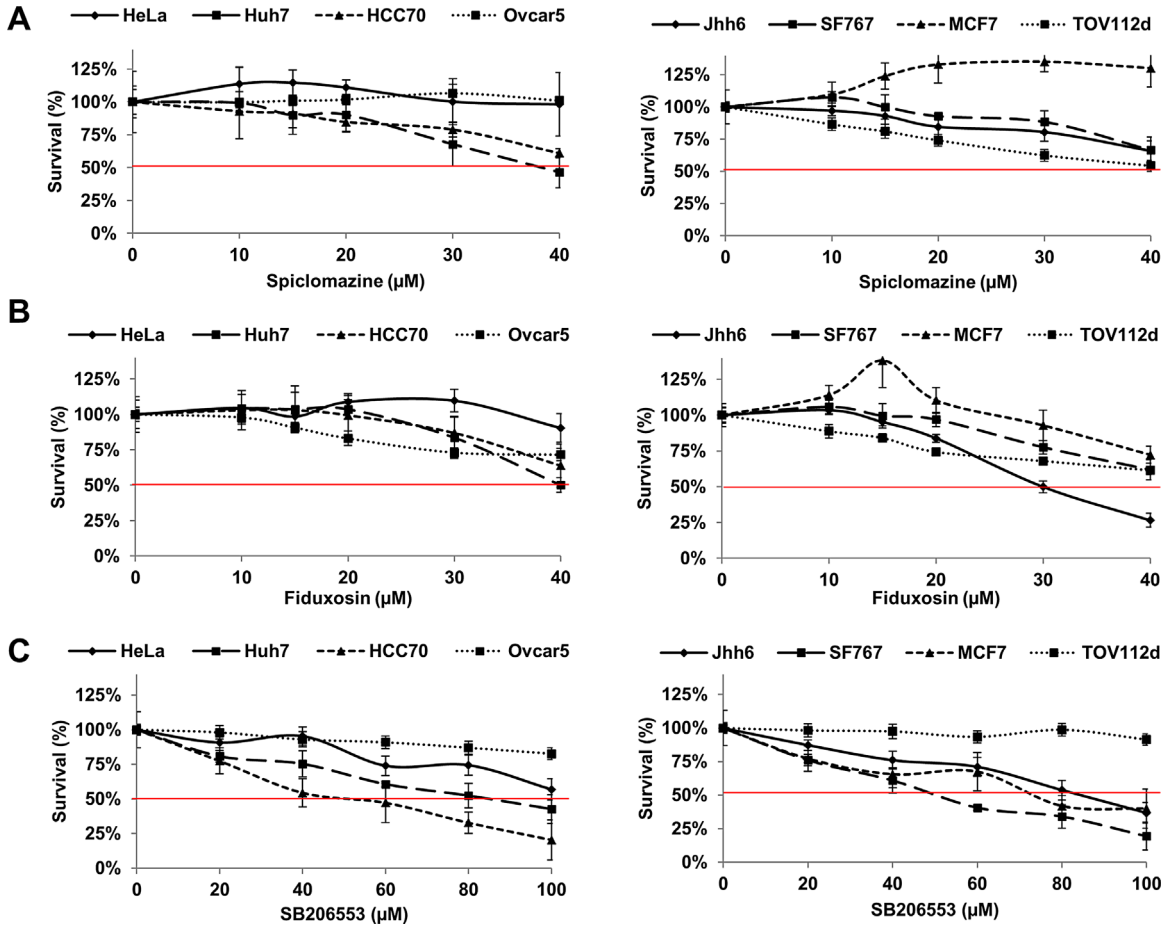


Figure 10. IC_{50} estimates for spiclomazine, fiduxosin, and SB 206553 on different tumor cell lines. Representative cytotoxicity curves used for the calculation of the IC_{50} of selected APE1/NPM1 inhibitors. The indicated cell lines were grown in presence of increasing concentrations of spiclomazine (A), fiduxosin (B) or SB 206553 (C) for 48 h; cell viability was measured through the MTS assay. Maximum dosage was limited by the poor solubility of fiduxosin and spiclomazine in aqueous medium. Values plotted are the average ± SD of five experimental replicates. 50% survival is highlighted by a red line.

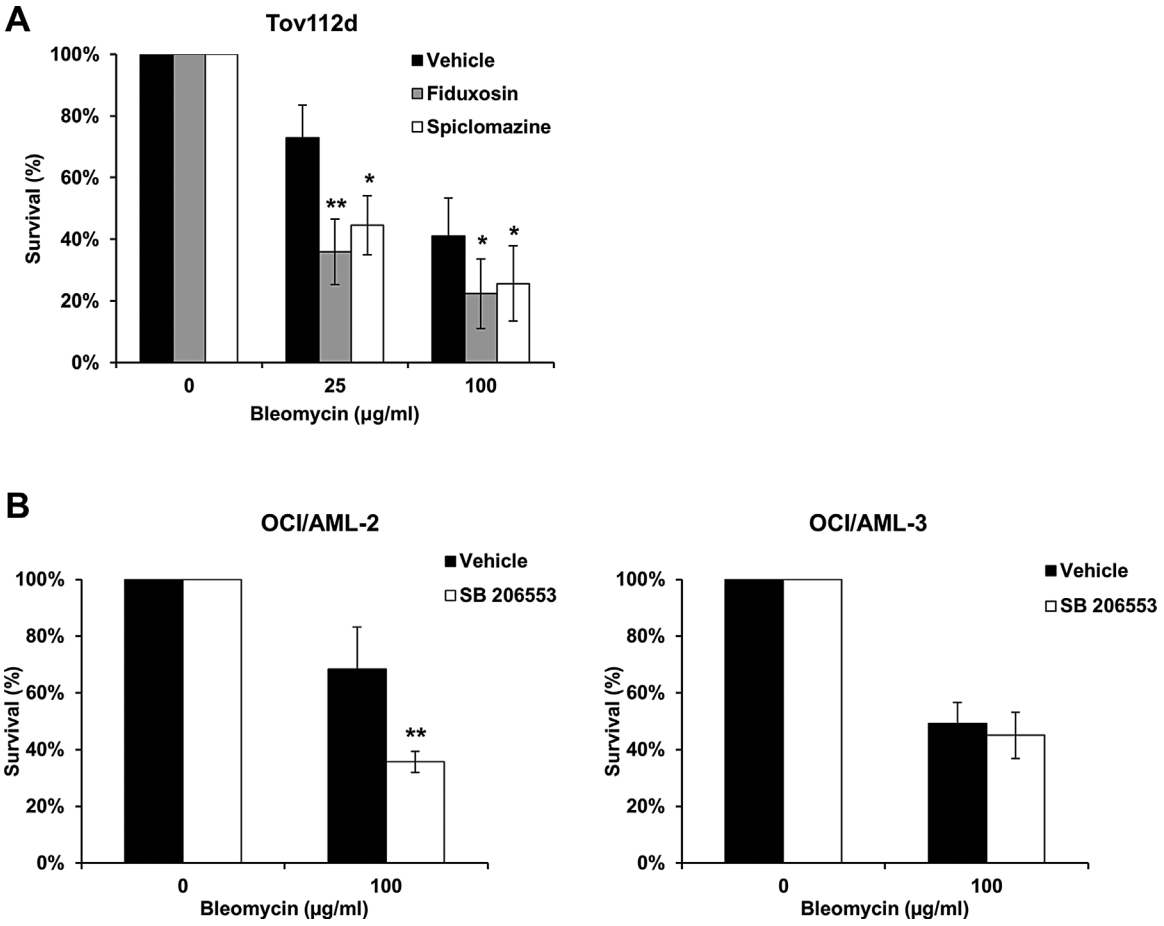


Figure 11. Spiclomazine and fiduxosin sensitize TOV-112D cells to bleomycin, while SB 206553 shows differential sensitization to bleomycin in AML cell lines. (A) TOV-112D ovarian cancer cells were incubated with 20 μM of either fiduxosin or spiclomazine for 7 h and subsequently challenged with increasing amounts of bleomycin for 1 h in presence of unchanged inhibitor concentration. Cell viability was monitored 48 h later through cell counting. The cell killing effect of fiduxosin and spiclomazine as single agent was, respectively, $0.98 \pm 0.07\%$ and $0.91 \pm 0.10\%$ relative to the vehicle. Data reported are the mean $\pm$ SD of at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$. (B) OCI/AML-2 or OCI/AML-3 cells were incubated for 24 h with 20 μM SB 206553, challenged with bleomycin (1 h, 100 μg/ml), washed and incubated with unchanged SB 206553 concentration for further 24 h. Cell viability was assessed through cell counting. Data reported are the mean $\pm$ SD of at least four experimental replicates, $^{**}P < 0.01$. The effect of SB 206553 relative to the vehicle as single agent was $1.40 \pm 0.06\%$ for OCI/AML-2 and $1.27 \pm 0.09\%$ for OCI/AML-3 cells.

the AlphaScreen® technology using full length recombinant proteins, we were able to screen a set of commercially available small molecule libraries for putative inhibitors of the APE1/NPM1 interaction. Although our data do not allow us to exclude the possibility of in vivo off target effects of the top-hits, using our proof of concept approach, we have successfully demonstrated that structurally unrelated bioactive molecules are able to impair the APE1/NPM1 interaction in living cells. Moreover, the inhibition of this interaction leads to interesting drug potentiation and growth impairment phenotypes in tumor cell lines.

Targeting protein–protein interactions with small molecules is a challenging task. The issue is often complicated by the presence of large surface areas involved in the protein–protein binding, and by the lack of obvious binding pockets for the small molecules at many interaction interfaces [41,42]. Since the APE1/NPM1 protein–protein interaction is known to involve the N-terminal region of both proteins [16,17], we speculate that the compounds identified herein are targeting either the NPM1 oligomerization domain, or the APE1 unstructured extension [18], or even the APE1/NPM1 interface. The propensity of many positive hits to induce relocalization of APE1 implies that this class of inhibitors has greater affinity for the N-terminal region of the endonuclease, which is responsible for its nuclear localization [43] and interaction with NPM1 and rRNA [16–18]. The negative effects measured on APE1 redox function and on the endonuclease activity of the enzyme, together with the SPR data, strongly support preferential binding to APE1. In this study we did not carry out structural analyses to pinpoint the precise binding site of the hit compounds on APE1.

Therefore, we cannot exclude that the small molecules may interact with different sites and/or affinities at the interaction interface. A preliminary observation of the molecular structure of the top hit compounds (Figure 5) suggests, for instance, that different chemical features of the molecules may affect their activity towards APE1. The experiment described in Figure 6C shows that both spiclomazine and fiduxosin inhibit APE1 with potency comparable to that of compound #3 [37]. Structure-activity relationship (SAR) studies have also shown that thiazolinic and thienopyridinic groups within compound #3 are associated with increased inhibitory activity of the molecule [37]. Interestingly, both fiduxosin and spiclomazine contain a thienopyrimidinic and thiazin moieties, presumably involved in the APE1 inhibition mechanism. The lower inhibitory effect of troglitazone and SB 206553 might be explained by (1) the presence of a less efficient thiazolinic moiety and (2) the absence of any sulfur-containing group. These features could explain the different behavior shown by molecules selected to target the same protein–protein interaction. However, these speculations need to be further addressed by targeted SAR investigations.

The impairment of the APE1 redox function might be, per se, an appealing feature for anti-cancer treatment, as already suggested [15], and a subset of the APE1/NPM1 inhibitors counteract the TNF-α-induced NF-κB activation in hepatocarcinoma cells. Among the positive hits in this study we found troglitazone, a well-known anti-diabetic PPARγ agonist that has been withdrawn from the market for its hepatotoxicity [44]. Notably, troglitazone has been proposed to exert anti-inflammatory action through the NF-κB pathway [45], and various reports have described interesting anti-tumor properties of this compound in different cell models [46,47]. These findings might be explained by our observation that the compound is a very potent APE1 redox inhibitor.

Polischouk and colleagues previously reported an interesting enhancing effect of the antipsychotic trifluoperazine (TFP) on bleomycin-mediated cytotoxicity [48]. One of the present APE1/NPM1 inhibitors, namely spiclomazine, is a member of the same class of drugs (i.e., phenothiazines), and we consistently observe here a synergic effect of bleomycin and spiclomazine. Although TFP has been suggested to impair the non-homologous end joining process [48], an involvement of either APE1 [40] or NPM1 [19] in the elimination of bleomycin-induced DNA damage should not be excluded. Interestingly, spiclomazine has recently been proposed as a selective molecule for targeting pancreatic carcinoma, being able to reduce proliferation of cancer cell lines, with minor effects on non-transformed cell models. In the same study, spiclomazine was proposed to regulate the expression levels of apoptotic proteins, reducing the mitochondrial membrane potential, elevating reactive oxygen species levels and suppressing the metastatic potential of pancreatic carcinoma cell lines [49]. The redox activity of APE1, moreover, has been shown to be an ideal key target in pancreatic cancer [15]. In light of our findings, we speculate that the dual effect of spiclomazine on both the APE1 endonuclease and its redox activity might be linked to the effects observed on pancreatic tumor cell lines.

In this study, we show that molecules already known for different biological activities display previously uncharacterized anti-tumor properties. The sensitization effects observed when disrupting the APE1/NPM1 interaction, in combination with MMS or bleomycin, appear to be only partially related to a direct impact of the compounds on the BER capacity. In fact, while the inhibitory effect displayed by some molecules toward the APE1 endonuclease and redox activities is correlated with a negative impact on cellular proliferation (compare for instance fiduxosin and spiclomazine with SB206553), our data did not record any significant DNA damage accumulation under the experimental conditions tested (with fiduxosin being a clear exception). This apparent contradiction could reflect the low sensitivity of the assays used, since the negative effect on the APE1 endonuclease activity is fairly limited in terms of magnitude, at least at the concentrations tested in vitro. On the other hand, it is conceivable that non-canonical APE1 activities, distinct from its DNA repair-related function (e.g., RNA binding/cleavage, exonuclease, or RNase [6]), are involved in the response to the DNA damaging agents used in our experiments. Notably, genotoxins tested within this study are likely to induce the accumulation of RNA damage, along with DNA damage [50–52]. These considerations reflect the possible relevance of the APE1/NPM1 interaction in pathways different from DNA repair. Accordingly, the observed anti-proliferative effect of some of the inhibitors (i.e., spiclomazine and fiduxosin) may reflect a potential role of the APE1/NPM1 association in tumor cell proliferation, but not specifically in DNA repair, as previously suggested [21]. The impairment of any potential RNA cleansing function of the APE1/NPM1 complex [17] without obvious accumulation of DNA damage might explain why these inhibitors are cytostatic and cytotoxic. In addition, it is worth highlighting that the APE1/NPM1 inhibitors were identified for their ability to induce relocalization of APE1; therefore, it is possible that some of the effects observed (including the reduction in redox activity of APE1) are provoked by a relocalization of APE1 itself.

A limitation of this study is represented by the fact that our data cannot completely rule out that the effect of the small molecules is mediated in part by the disruption of the interaction between APE1 and other protein partners. It is clear, from our SPR data, that the N-terminal extension of APE1 is required for the

interaction with two of the inhibitors. However, further interaction experiments exploiting the APE1 NΔ33 and the APE1 KA mutants to address the specificity of the inhibitors are not feasible. The results of the experiments, in fact, would be biased in that both the mutant proteins display increased AP-endonuclease activity, impaired RNA binding and reduced binding activity toward known APE1 binding partners, as already reported [16,17,21]. To the best of our knowledge, however, our study represents the first example of the selection of low molecular weight protein–protein inhibitors targeting the flexible N-terminal region of APE1. It is worth underlining that the compounds identified in this study were screened using commercially available small molecule libraries, in the absence of any structure-based design. Further SAR studies will have to be carried out in order to improve the selectivity and specificity of the mechanism of action of these molecules.

In conclusion, these results show that bioactive molecules selected for their ability to impair the APE1/NPM1 association within cells are able to synergize with therapeutically relevant DNA damaging agents, increasing their cytotoxicity. Moreover, some of the molecules show interesting anti-proliferative activity as single agents. The anti-cancer properties of these molecules and their mechanism of action deserve further characterization; additional studies aimed at the improvement of the APE1/NPM1 inhibitors as novel therapeutic compounds are warranted. Design of more potent inhibitors might be useful for a thorough characterization of the relevance of this protein–protein interaction in vivo, as well as to improve existing therapeutic approaches in combination therapy.

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