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BASE EXCISION REPAIR

IN THE MAINTENANCE OF GENOME STABILITY

IN NEURONAL CELLS:

NEW INSIGHTS FROM CADMIUM AND CISPLATIN

TREATMENT STUDIES

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1 Preface

DNA repair proteins play an essential physiological role in the maintenance of genome integrity, being crucial to ensure cellular viability and proliferation (1). Therefore, recently, scientific interest is increasing around the possibility to target DNA repair pathways in order to modulate the effects of different cytotoxic compounds, possibly enhancing the efficacy of chemotherapeutic treatments or regulating the development of neurodegenerative processes (2,3).

Normally, DNA results constantly threatened by damaging agents, exogenous (such as by heavy metals as cadmium) or endogenous, inducing alterations to the normal sequence and/or structure of DNA bases. These damages affect both cell cycle progression and genome stability potentially leading to cell death or to the onset of cancer transformation because of the establishment of specific mutations (4,5). To counteract this high genomic instability, the organisms have developed different repair pathways that, through the coordination of the activities of several DNA repair proteins, allow the recognition and the removal of various types of lesions restoring the correct sequences (6,7).

Although some physiological processes, as aging and neurodegeneration, could affect the efficacy of these repair mechanisms thus promoting an increased cellular susceptibility to damaging agents (8), indeed alteration or inactivation of DNA repair pathways have been reported also in many types of cancer cells (9–11). Interestingly, in the development of both pathologies, namely neurodegenerative diseases and tumors, an important role is played by oxidative stress conditions, potentially inducing DNA oxidative damages (12–15) promptly processed through the Base Excision Repair (BER) pathway (12,16) involving DNA glycosylases, AP endonucleases, DNA polymerases and ligases (16).

Usually, the most common damage removed by the BER pathway is represented by base oxidation induced by the attack of reactive oxygen species (ROS) (12), occurring as a consequence of perturbation of the intracellular redox homeostasis caused by endogenous- or exogenous-mechanisms (17). Often, to promote this condition, other cellular systems devoted to the maintenance of redox homeostasis, as antioxidant defenses or the same BER pathway, are compromised or inhibited, thus enhancing the amount of alterations induced by ROS-exposure (16–18).

It has been reported that neurological disorders are strictly related to the coexistence of a decrease efficacy of DNA repair pathways, as BER mechanism, and an increase amount of ROS (14,19,20). Similarly, in the development of cancers, many reports point out the contribution of oxidative stress in both promotion and progression of the carcinogenesis process, further underpinned by an impairment of the antioxidant defenses (13,15). In addition, the proliferation of cancer cells is ensured by pre-replicative DNA repair mechanisms, exploiting, for example, the BER pathway (3), resulting particularly relevant also in cancer resistance phenomena (21–23).

Starting from these evidences, BER proteins play and essential role in the onset and/or development of both pathologies. Therefore, given the key role played by BER mechanism both in promoting neurodegeneration processes but also in ensuring cancer cell progression and chemoresistance, this repair pathway has now emerging as potential target to cope the development of these diseases, particularly enhancing sensitivity to cancer therapy (2,3).

In this context, the main mammalian AP endonuclease, the Apurinic/apyrimidinic endonuclease-1 (Ape1) can represent a good candidate.

Notably, this protein plays a key role in the BER pathway, by cleaving the AP site (24) formed upon removal of the damaged base, thus allowing the consequent fulfillment of the repair. Moreover, besides this crucial role in DNA repair, Ape-1 has at least another important activity: indirectly, it contributes to the cellular adaptive response induced by oxidative stress, regulating the expression of specific genes through the redox activation of their transcriptional factors. To support the high potential of targeting Ape1, alteration of its functions and/or expression levels, localization or sequence (e. g. polymorphisms) have been associated to the onset of neurodegenerative diseases as well as to the development of different types of tumors, in particular to their aggressiveness and resistance to therapeutic treatments (25–27).

Although the importance of Ape1 functions is well established in many tissues (22,28), its role in the pathologies of nervous system, included neurodegeneration and cancers, could be more relevant and complex although, to date, it is not well defined.

In this tissue, in fact, the high levels of reactive oxygen species (ROS) as byproducts of the big physiologic consumption of oxygen and metabolism but also as result of the action of exogenous factors (environmental pollutants, side effects of chemotherapeutics agents,…) promotes a potential increase of oxidative DNA

damages (19), thus possibly enhancing neurodegeneration and cancer development (13,19).

Therefore, in this context, Ape1 could represent the ideal target to counteract the processes of neurodegeneration as well as to sensitize to chemotherapeutic treatments of nervous cancer, as gliomas, particularly resistant to therapy (29). For this reason, a good knowledge of the involvement and the role of Ape1 functions in response to different toxic agents could help to specifically target the protein activity in order to protect or to sensitize the neuronal cells to genotoxicants.

To explore the above mentioned unknown aspects, this work of Thesis aims to dissect the importance of the two principle Ape1 functions, DNA repair and redox activity, in neuronal cells and specifically in response to two toxic agents. Notably, these genoxicants have been carefully chosen to induce, directly or indirectly, oxidative stress conditions and specifically because of their association either with neurodegenerative processes, like the heavy metal cadmium classified as environmental pollutant (30), or with chemoresistance phenomena, as the crosslinking agent cisplatin used in cancer treatment causing also peripheral neuropathy (21,31).

Using SF-767, a glioblastoma cell line, treated with Ape1 inhibitors, specifically blocking either the DNA repair (Methoxyamine (32), compound #3 and compound #52 (33)) or the redox (E3330 (34,35)) activity, it has been observed a dysregulation of the other BER proteins, in particular of DNA polymerase δ, only in presence of a direct inhibition of the endonuclease activity of Ape1, occurred using the compound #3 or #52. To test the relevance of a possible feasibility of these inhibitors to modulate the cytotoxicity of genotoxic agents, these compounds were used in combination with cadmium or cisplatin.

Nevertheless, differently from what expected, an opposite combinatory effect was obtained adding Ape1 direct endonuclease inhibitors to these genotoxic treatments, probably due to the distinct role of BER mechanism in response to these type of damages. Moreover, although both cadmium and cisplatin similarly affect the expression levels of polymerase δ, this effect has been related to the impairment of a specific though different cellular mechanism involving polymerase δ. In addition, since both cadmium and Ape1 inhibitors induce a similar BER alteration, specifically of polymerase δ, the possible regulatory mechanisms explaining its reduction has been analyzed. Although further experiments are necessary to better define the

molecular mechanisms involved in this modulation, first results point out that cadmium and Ape1 inhibitors impact on polymerase δ expression levels through different mechanisms.

2 Introduction

2.1 DNA repair pathways

DNA is continuously subjected to the action of endogenous and exogenous agents that could potentially damage it. The endogenous lesions include oxidation, alkylation, inter or intra strand adducts and hydrolytic depurination or deamination (4) that can be induced also by exogenous agents, such as environmental insults (chemicals, UV light, pollutants, carcinogens) and by the action of chemotherapeutic agents (drugs and radiation) (1). It is estimated that in each cell, up to ten of thousands of DNA-damaging events may occur *per day* (36) seriously threating the genome integrity thus interfering with the physiological cellular processes and compromising the cell viability. Moreover, in specific DNA regions, failure of the repair mechanisms can potentially lead to the onset of mutations harboring to different diseases included cancer and aging (37). As a consequence, to preserve genome integrity and stability, cells have developed a complex network of DNA repair mechanisms that, although the same lesion can be recognized by more than one pathway, are specialized in the removal of specific DNA damages. Nevertheless, regardless of the type of lesion, all the repair mechanisms are activated by a common cascade of events, called DNA damage response (DDR). Notably, DDR may detect the lesion and signal downstream its presence to promote and coordinate its removal through the DNA repair systems: as a result, these two events are not totally separated but rather they co-operate together sharing some components (38). DNA repair pathways involve a lot of proteins which number is further increasing, as well as the knowledge of the mechanisms and the molecules regulating them (7). Alterations of DNA repair proteins or mechanisms represent contributing factors to the onset of different pathologies and are associated to the development of neurological disorders (20).

Genome lesions may cause mainly single strand breaks (SSB), double strand breaks (DSB) and alterations of both DNA bases or sequence (oxidation, hydrolytic depurination or deamination, insertions, deletions, mismatches) and of the double helix DNA structure (e. g. formation of covalent adducts).

Although the chemical modification of bases can be repaired by a single step mechanism called direct protein-related reversal (DR), the other pathways require a sequence of catalytic reactions to solve more complex damages (Fig. 1).

Nucleotide excision repair (NER) pathway ensures the removal of inter or intra-strand adducts causing a distortion of the double helix of DNA, while the mismatch repair (MMR) detects errors arising from DNA replication, such as insertion, deletion and mispaired bases, restoring the correct sequence (8). To solve DSB, that is the most harmful DNA damage involving both DNA strands, cells can exploit two different mechanisms: the non-homologous end-joining (NHEJ) and the homologous recombination (HR) pathways (37,40–43). In the NHEJ the two ends are reconnected without the use of homologous sequences thus leading to a consequent deletion of DNA regions, while in the HR the homologous and undamaged DNA molecules may act as template to repair the broken one. On the other hand, the SSB repair and the base excision repair (BER) pathways share the last three steps: notably, in BER the introduction of a single strand break is a key phase of the repair mechanism, which is essential in order to restore the correct sequence (6,16,40).

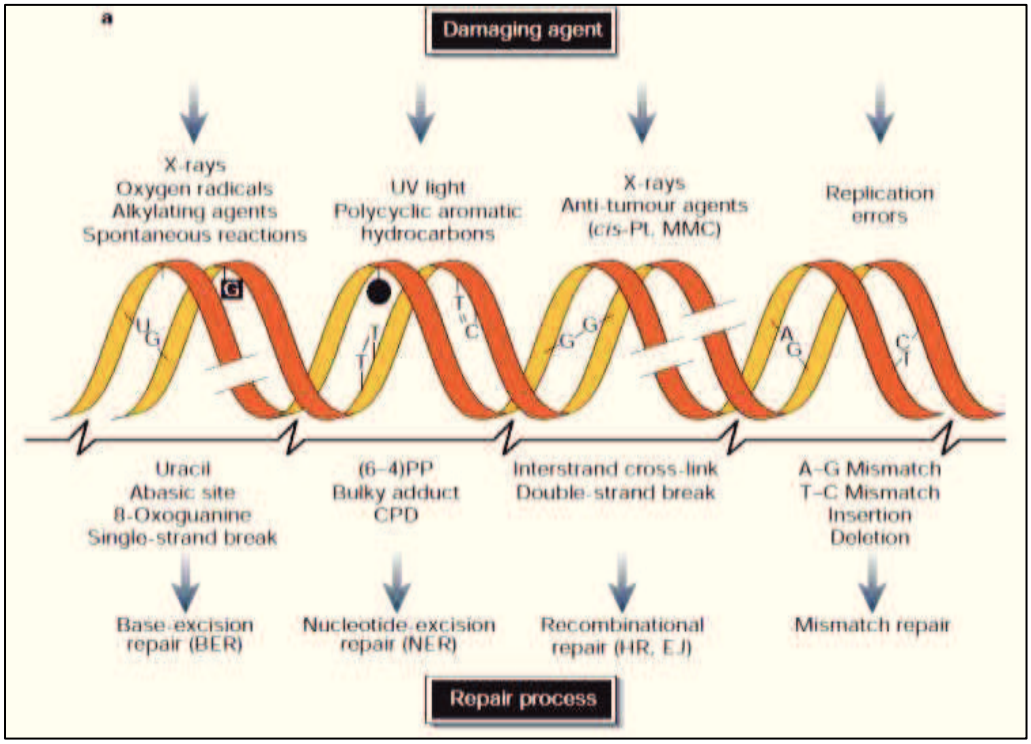

Figure 1: ***DNA repair pathways*****.**

Several repair mechanisms are involved in the maintenance of genome stability, including Base Excision Repair (BER), Nucleotide Excision Repair (NER), Direct Reversal (DR), Mismatch Repair (MMR), Homologous Recombination (HR) and Non Homologous End Joining (NHEJ). Each of these pathways is required for the repair of the lesions reported in this figure (Image adapted from: Hoeijmakers, *Nature* 2001).

2.2 Base Excision Repair pathway

It is estimated that generally more than 10^4 base lesions may affect single strand DNA daily in mammalian cells (36). Damages without significant distortions of the double helix structure are typically repaired by the evolutionary conserved BER pathway and include oxidation as well as spontaneous alkylation of base, formation of AP-sites and deamination (44). Although BER mechanisms consists of two sub-pathways, called *short-patch* and *long-patch*, the four key reactions required to repair the lesion are the same and include: damaged base excision, AP site cleavage, gap filling and nick ligation. The main difference between the two sub-pathways is related to the gap filling step: notably, the damaged base can be replaced, respectively, either with the only correct nucleotide or with an upstream stretch of some nucleotides. Despite the mechanisms responsible for the choice of which of the two pathways is poorly understood, several hypotheses have been suggested but the most reliable one ascribes the decision to the nature of the intermediate produced upon AP site processing and its removal (45).
Anyway, both BER sub-pathways (*short and long patches*) use the following main steps (Fig. 2) requiring the same classes of DNA repair proteins (DNA glycosylases, AP endonuclease, DNA polymerases, and DNA ligases).

a) *Base excision and cleavage of the AP-site*

   The repair process starts with the recognition and the removal of the damaged base by DNA glycosylases. This class of proteins includes several enzymes acting on different subtypes of lesions, even if these subsets can be somehow overlapped. DNA glycosylases can be monofunctional or bifunctional and in both cases they cleave the N-glycosidic bond exciding the base and leaving an abasic site (AP-site) on DNA (46,47). The second reaction requires the endonuclease activity of the Apurinic/apyrimidinic endonuclease 1 (Ape1) that, upon recognition of the AP site, cleaves the intact sugar phosphate backbone. The single strand break generated presents a 3'-OH end and a transient 5' deoxyribose phosphate (dRP) terminus that blocks the following gap-filling reaction (48).

b) *5' SSB end processing and gap filling*

To allow the insertion of the correct base by DNA polymerases, it is necessary to remove the 5' dRP residue, generated by the Ape1 cleavage activity. Notably, the two BER sub-pathways, the *short* and *long-patch*, can be distinguished depending on the reactions used to this purpose.

In the *short-patch*, that represents about 80-90% of all BER processes, the DNA polymerase β (Polβ) itself is able to remove the dRP residue, through its 5' dRP lyase activity (49). Then, by using the complementary strand as a template, Polβ adds the correct base, filling the single nucleotide gap remaining in the lesion site and replacing the correct nucleotide sequence.

On the other hand, in the *long-patch* pathway the dRP is displaced with some other additional nucleotides present upstream the damage site, usually of three to eight, forming a single strand DNA flap that is finally removed by flap endonuclease 1 (FEN-1), an enzyme normally involved in DNA replication (50). Although Polβ may be recruited to incorporate nucleotide also in this sub-pathway (51), usually the *long-patch* exploits other proteins normally used by the DNA replication machinery, included DNA polymerase δ/ε (Polδ, Polε), the sliding clamp Proliferating Cell Nuclear Antigen (PCNA) and the replicator factor-C (RF-C) (45,52,53).

c) End sealing by DNA ligase

Upon nucleotides incorporation, regardless of the sub-pathway involved, a nick with 3' OH and 5' phosphate ends remains in the DNA backbone. Thus, in the final step, in order to completely restore genomic integrity, cells use DNA ligases to seal the strand nick. Ligase I and ligase IIIα are the main enzymes employed respectively in the *long* and *short-patch*, even if this separation is not so strict (54).

Besides these enzymes, other accessory proteins are recruited in the BER pathway to allow a fine regulation and coordination of all DNA repair proteins involved. One of these is the poly(ADP-ribose)polymerase1 (PARP-1), a sensor of DNA strand breaks, that detects and binds them by recruiting other proteins to enhance the total efficiency of the repair processes (55). In the *short-patch* of BER, also X-ray cross complementation group1 (XRCC1) is immediately recruited by the repair complex, upon cleavage of the AP-site. Nevertheless, since it has no demonstrated enzymatic activity, XRCC1 acts as scaffold protein playing a key role to coordinate the final

steps of the repair through the *short-patch* pathway. In particular, it recruits and interacts with Polβ (56) and Ligase IIIα (57) stimulating their activities and bringing them to the same site of repair. On the other hand, the same scaffold protein role is played by PCNA (58,59) in long-patch BER. It enhances the processivity of Polδ and stimulates FEN1 activity (51,60) promoting the choice of long-patch pathway.

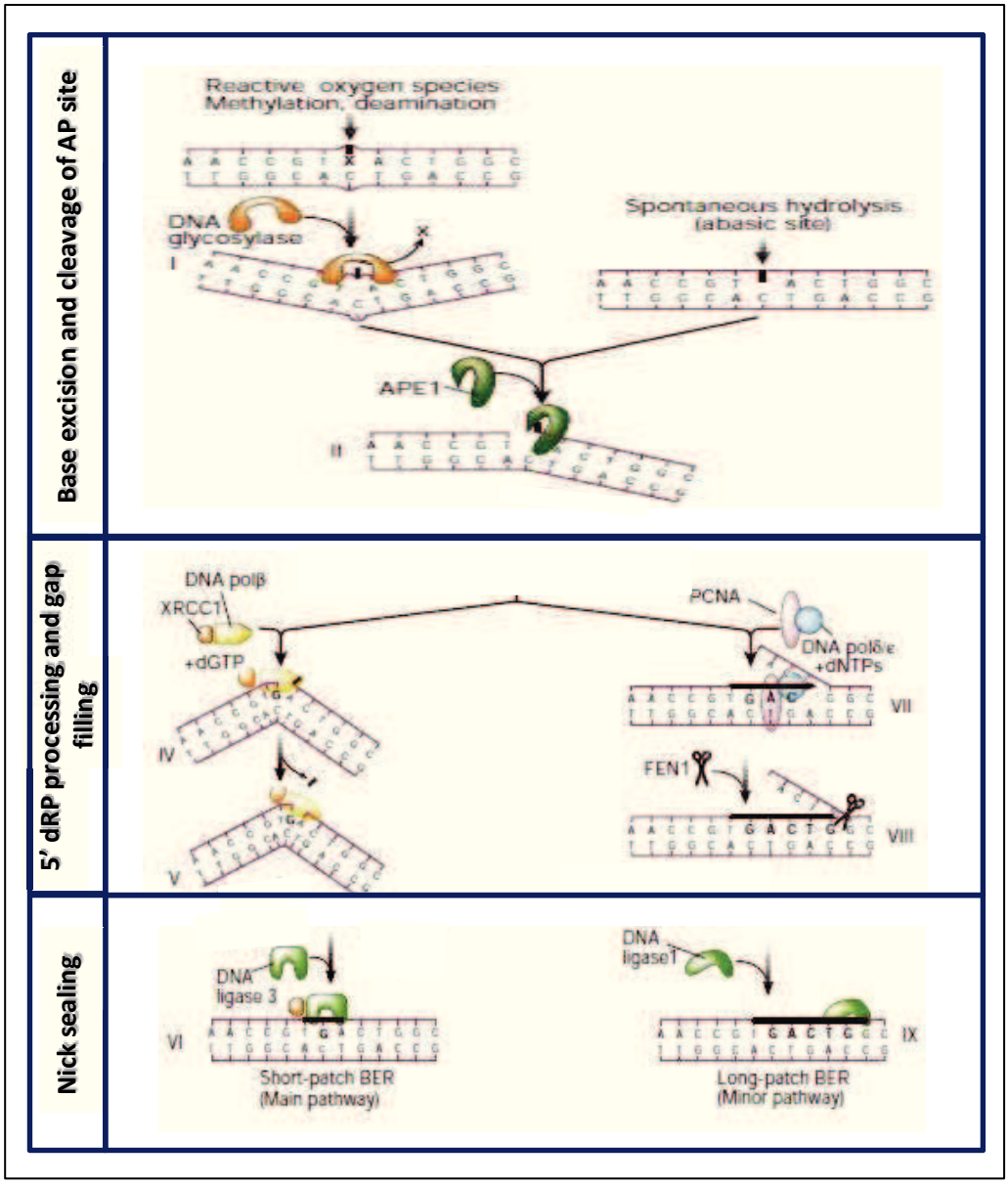

Figure 2: *BER pathways*.

Schematic illustration of the three main phases of BER pathway, with a representation of both subpathways, short and long patch (Image adapted from Hoeijmakers, *Nature* 2001).

Moreover, all the enzymes involved in the different BER steps may mutually interact to provide a correct repair of the damage through a fine regulation of each distinct phase.

2.2.1 DNA damages repaired through BER pathway

The main role of BER is the repair of damages occurring to DNA bases through their excision and substitution. DNA bases lesions can be induced by intrinsic or extrinsic factors that, essentially, may lead to three types of modifications: oxidation, alkylation and deamination.

Base Oxidation

Oxidative damages occurring in cells are caused by the action of ROS that are constantly present in the intracellular space (Fig. 3), both as byproducts of physiologic and enzymatic mechanisms, such as respiration, and upon induction by the activity of some exogenous agents. Despite cells have developed several mechanisms to cope with ROS effects, these species become dangerous when their amount exceeds the capability of these antioxidant defenses. Oxygen radicals include: superoxide anion (O_2^-) produced by oxidases and respiration; hydrogen peroxide (H_2O_2), that is generated through enzymatic processes; hydroxyl radicals ($OH^\cdot$) generated as byproduct of Fenton reactions and singlet oxygen ($^1O_2$). All these species may react with several cellular molecular substrates, such as proteins, lipids and DNA. Notably, over one hundreds of different oxidative DNA adducts are related to ROS effect, included bases oxidation, AP-sites formation (generated by enzymatic processes during the repair mechanism or by spontaneous hydrolysis of the N-glycosidic bond) and single or double-strand breaks (17). Similar modifications can be induced also by endogenous reactive nitrogen species, first of all nitric oxide (NO·) and its byproducts (61) (Fig. 3).However, the efficient repair of these lesions is essential to preserve both the viability of the cells and the integrity of the genome: in fact, while unrepaired double strand breaks represent a lethal damage for the cells, base modifications (such as oxidative lesions) could generate mutations and/or may exert cytotoxic effects. All bases can be targeted by ROS (Fig. 4): while oxidation of purines bases mainly form 8-oxoguanine (8-oxoG) and formamidopyrimidines (derived from guanine or adenine adducts, respectively FapyG and FapyA) (62), the most common products of pyrimidines oxidation are thymine glycol and 5-

hydroxyuracil (5-OHU), that may be detected in comparable amounts to 8-oxoG in human DNA (63).

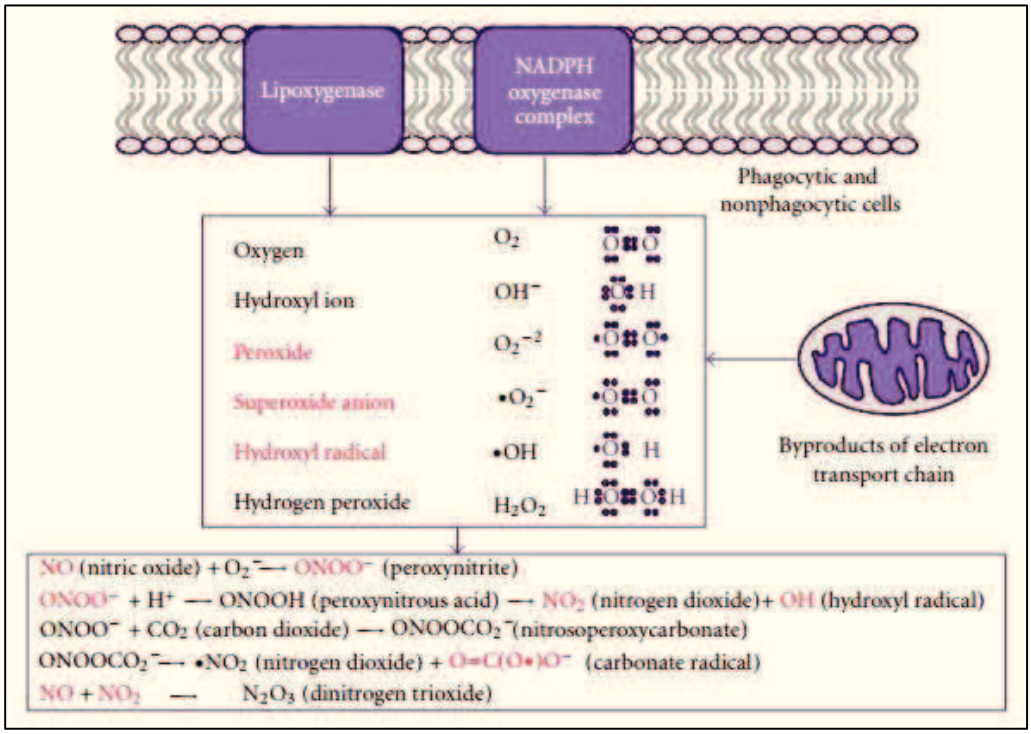

Figure 3: ***Reactive species of oxygen and nitrogen.***

Main sources of reactive oxygen species production are reported in the figure and include the mitochondrial respiratory chain but also the activities of NADPH and lipoxygenase. Oxygen free radicals can react with NO generating reactive nitrogen species. In red are evidenced species highly reactive because of the presence of unpaired electron (Image adapted from Deavall et al, Journal of toxicology 2012).

Notably, the most studied oxidized base is guanine, whose modifications, if not repaired, may lead to the onset of mutations: in particular, the 8-oxoG may cause a transversion GC:TA due to its preferential pairing with adenine rather than cytosine during DNA replication processes, while the formamidopyrimidine guanine adducts may generate a transversion GC:CG (62). On the other hand, also some pyrimidinic lesions are potentially mutagenic: beside the 5-hydroxycytosine (5-OHC) that is considered the most mutagenic of the oxidative DNA damages (64), also other modifications exert the same mutagenic effect, such as 5-hydroxy-2'-deoxycytosine and 5-hydroxyuracil that provoke a transition GC:AT and a transversion GC:CG (65), respectively. Despite the other oxidative lesions are not implicated in the onset of genome mutations, some of these may interfere with specific cellular mechanisms: for example, thymine glycol can cause a weak mutagen transition C-T (66,67) able to

block transcription and replication (68), while the oxidation of the 5-methyluracil alters the binding of transcription factors to DNA (69) modifying the gene expression.

Base Alkylation

Another type of genome damage is represented by the addition of alkyl group to DNA bases through a process named alkylation. The donors of alkyl groups can be endogenous or exogenous molecules. These second ones are usually used as therapeutic agents to limit the development of cancer, given their toxic effect on cell viability. Among the endogenous alkylating molecules, the most well-known is the donor S-adenosylmethionine (SAM), even if alkyl radicals can be derived also from lipid peroxidation (methyl radicals) (70,71). Nevertheless, SAM remains the main endogenous donor of methyl groups used in cellular reactions, where the methyl radicals can be accepted by several biological molecules. Anyway, given the high transfer potential of SAM, the addition of this group could also occur spontaneously without the occurrence of specific enzymatic reactions and may usually involve nucleic acids and proteins (72). Although the alkylation induced by SAM is 2000 fold weaker compared to the effect of methyl methanesulfonate (MMS), an exogenous experimental alkylating agent, the effects obtained on DNA molecule are the same (70). Modifications induced by SAM mainly occur on double strand DNA and involve purine bases specifically producing 7-methylguanine and 3-methyladenine (3meA), while other alkylating agents may act on single strand DNA producing 1-methyladenine (1meA) and 3-methylcytosine (3meC) (73,74).

Even though these lesions are not potentially mutagenic, they may differently affect cell viability, in fact while guanine methylation is an harmless modification, adenine alkylating lesions have a strong toxicity. Despite pairing with the other strand is not altered by its methylation, the alkyl group of this modified adenine, taking up the minor groove of the double helix, may interfere with the activity of RNA and DNA-polymerases, probably justifying the low mutagenicity of these type of lesions (72).

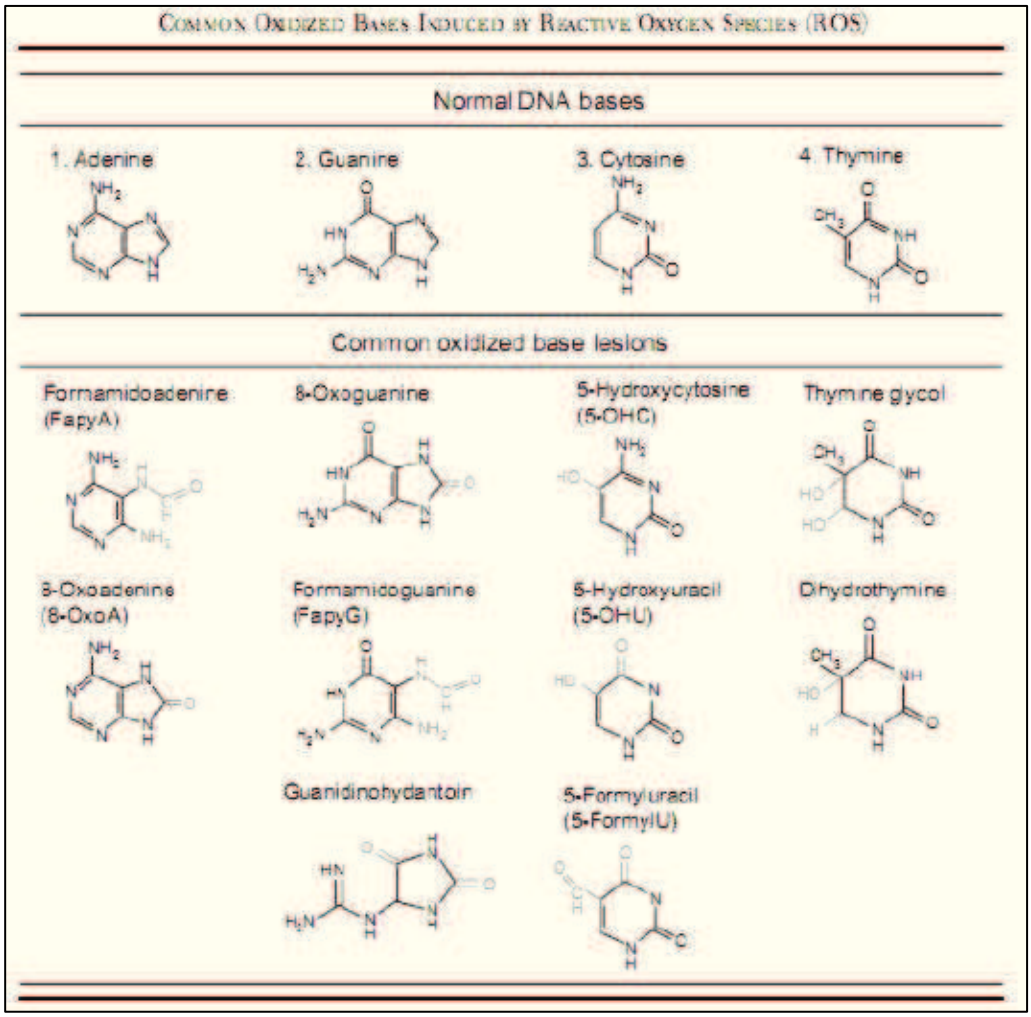

Figure 4: ***DNA bases and their oxidation*****.**
Chemical structures of normal and oxidized bases (Image from Hegde et al, *Mechanisms of Ageing and Development* 2012).

Base Deamination

Despite uracil is not a constitutive base of DNA, it can frequently be found in the DNA structure as a result of the hydrolytic deamination of cytosine. It has been estimated that deamination events occur more frequently on pyrimidines than of purines and in single stranded DNA rather than in double stranded. The main targets of deamination are cytosines and hundreds of them daily undergo to spontaneous hydrolytic deamination generating uracil base (4). The presence of this abnormal base is usually recognized and removed by several DNA glysosylases, such as SMUG1 (75), allowing the sequence to be repaired through the BER pathway. Whether this type of lesion is not removed before DNA replication, it may potentially induce a genomic mutation because of uracil similarity to thymine that pairs with an adenine inserted in the complementary strand ultimately leading to a transition from C:G to T:A (76). The

same result can be obtained whether the deamination occurs on an alkylated cytosine (5-methylcytosine) generating a thymine: although this base can be recognized and removed through a specific DNA glycosylase, its normal presence in DNA could not allow the identification of this lesion. In fact, the lack of repair of the damage caused by deamination of the 5-methylcytosine into thymine, before DNA replication, may generate a point mutation in the new strand, in which an A is substituted to a G (4). This type of lesion is the most common mechanism leading to mutation in DNA and is often observed in cancers. Nevertheless, even if less frequent, also guanine and adenine can be deaminated thus forming xanthine and hypoxanthine, respectively. In both cases, the new bases may induce a transition mutation, because of the pairing of xanthine with thymine rather than cytosine and hypoxanthine with cytosine, in place of thymine.

2.2.2 Damages activating BER pathway

The base modifications recognized by BER proteins can be induced both by endogenous and also by exogenous molecules that could differently alter the normal bases structure of DNA.

Endogenous damages

In aerobic organisms, oxidative stress is one of the main activator of BER pathway.

The physiological cellular metabolism is considered a source of reactive oxygen species (ROS), because of the final step that includes the oxidative phosphorylation during mitochondrial respiration. In this process, about 3% of the molecular oxygen is reduced to water through a continue transfer of electrons across the electron transport chain (77–79). Nevertheless, during this mechanism, it is possible that some oxygen atoms do not undergo complete reduction with the consequent formation of ROS intermediates (79). In order to solve this problem, aerobic cells have developed different specific enzymes that may further reduce these molecules, either directly to water molecules (catalase, glutathione peroxidase) or to other intermediates (superoxide dismutase), depending on the initial substrate (18).

Despite the presence of these antioxidant systems evolved to counteract the toxic effects of ROS, other enzymes may catalyze the generation of these reactive species (NADPH oxidase). Phagocytic cells, in particular neutrophils, exploit the toxicity of

ROS releasing them to destroy both exogenous organisms and also infected cells, although their toxicity may also hit normal cells (12,80).

Beside oxygen also other elements, such as nitrogen, can form free oxidant radicals. In particular, the most diffuse reactive nitrogen specie (RNS) is nitric oxide (NO) that can be produced and released by macrophages along with other ROS species (81).

In addition to these molecules, leading to oxidative damages, also base alkylation are removed by the BER pathway. Remarkably, to date, the only endogenous molecule responsible for the higher transfer of methyl groups is represented by the physiological donor S-adenosylmethionine (SAM), that may also lead to spontaneous methylation of biological macromolecules, such as DNA (72).

Lastly, also the spontaneous deamination of cytosine, that is highly instable, in uracil is recognized by BER proteins.

Exogenous damages

Although the generation of both oxidative and alkylating lesions may arise from the action of intracellular molecules, also exogenous agents are able to induce the same cellular damages (Fig. 5). Notably, the induction of bases modifications previously described (82), thus can potentially enhance the accumulation of DNA damages and, joined to the interference of several drugs with the different repair systems, can promote the possible onset of mutations.

Therefore, the association between exogenous agents, usually of environmental origin, and development of a variety of diseases, included cancer, arthritis, cardiovascular dysfunctions but also neurodegenerative pathologies and aging processes, was not a completely unexpected discovery.

Despite almost of these exogenous agents are present in the environment to which we are normally exposed, such as ionizing radiation, ultraviolet (UV) light and environmental pollutants (cigarettes smoke, traffic pollution, pesticides,…), other compounds causing the same DNA alterations have been chemically sensitized as strategy for anticancer therapies.

The target of these molecules, named chemotherapeutics, is to deliberately increase the damages within the tumor cells causing their death. However, usually the effect of these compounds is not only restricted to cancer cells but they may also lead to noxious side effects on remaining normal tissue.

Nevertheless, in order to better understand the study presented in this work, an overview of knowledge about two specific exogenous agents able to induce the activation of BER pathway does follow.

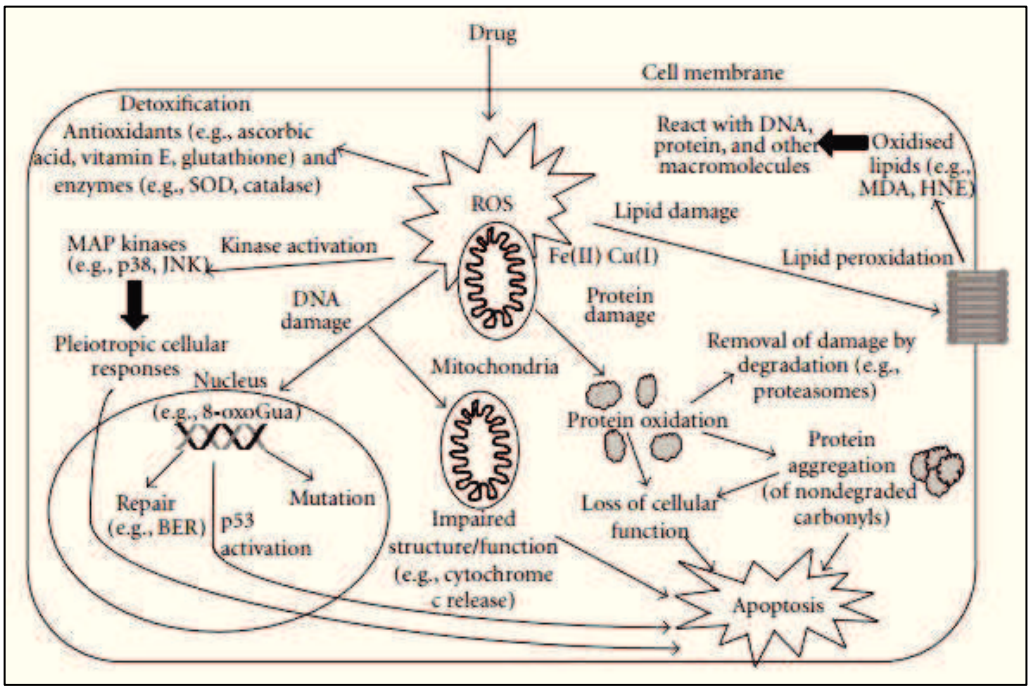

Figure 5: ***Cellular effects of oxidative stress induced by drugs*.**

The induction of an increased load of intracellular ROS may damage DNA, lipids and proteins. This general dysregulation promote the activation of signaling usually leading to cellular apoptosis (Image from Deavall et al, *Journal of toxicology* 2012).

Cadmium as environmental pollutant

Cadmium is a transition metal belonging to the IIB group of chemical elements. It appears as a white-silver powder and is considered one of the most dangerous heavy metals both for human and for environmental health.

The release of cadmium into the biosphere can derive from both natural and anthropogenic sources. Cadmium is a constitutive element of the Earth crust and its mobilization can be induced by the activity both of volcanoes and of weathering on the rocks, leading respectively to cadmium increase in air or soil and water. In addition, also forest fires and burning coal represent an important source of cadmium release. Nevertheless, a great increase of this metal pollution has been recorded afterwards the second world war, with the development of the industries. In particular, among these, the major anthropogenic source of cadmium derived from those devoted to the production of non-ferrous metal, causing its emission to air as by-product of mining, smelting and refining processes. Additional small amounts of

cadmium are released to air as dust formed during iron and steel scrap recycling (83). Although to date these systems again contribute to the release of cadmium, with technology development other anthropogenic cadmium sources have been identified. In particular, an high contribution derives from the disposal of batteries, usually containing 5 grams of cadmium, and waste or plastic materials incineration.

In highly industrialized regions, levels of cadmium in the air is about 500 $\mu g/m^3$ for week, while in rural areas with a sharply reduced pollution, the concentration is about of 300 $\mu g/m^3$ on average per year. This strong difference can explain also the high amount of cadmium in the soil near industries that is about of 1 µg/g than the limit established of 0.3-0.6 µg/g (84).

As a matter of fact, an high concentration of cadmium in the air leads to a major deposition on the agriculture top soil, with consequent absorption by plants thanks to its water solubility and chemical similarity to zinc. These cadmium amounts are in addition to the quantity acquired through pesticides, fertilizers and water used for irrigation that directly absorbs cadmium from the air. As final result, this accumulation in plants causes an increase of cadmium content also within foodstuffs, reflected in a major human intake. Other potential sources of cadmium for humans are claims, that absorb heavy metals from water sea usually contaminated both by industrial and by municipal discharges, and water ingestion because of its addiction to metals coating the pipes.

Nevertheless, despite cadmium can be absorbed through the gastrointestinal tract, inhalation of this atmospheric metal represents the major route of exposure for humans. To confirm this, experimental data demonstrated that about 50% of inhaled cadmium is absorbed through lungs, while only the 5% of that derived from the oral intake crosses the gastrointestinal mucosa. In non-polluted areas, average daily intake of cadmium from food is about 10-40 µg compared to the several hundreds of micrograms *per day* of the polluted areas (83) and the World Health Organization (WHO) has established a provisional tolerable weekly intake of 7 µg/kg body weight (85).

Anyway, non-smoking humans are unintentionally exposed to these sources of cadmium, but the absorption strongly increases in heavy smoking because of the accumulation of this metal in tobacco plants from the soil. As consequence, cigarettes can contain up to 1.2 µg of cadmium which about the 10% is absorbed through smoking. Furthermore, besides these non-occupational expositions leading to a gradual increase of cadmium levels with age, in occupationally exposed people

this amount increases more rapidly. Notably, these persons are employed in metal smelters, in the production and processing of cadmium and its alloys and compounds, in the recycling of electronic waste or in the production of particular pigments in paintings industries and, usually, the absorption derives from inhalation of cadmium powders.

Cadmium is considered a non-essential metal, as it is not necessary for physiologic cellular processes. Its toxicity for humans has been observed for the first time in 1955 in Japan: many people was poisoned by cadmium derived from mines located near inhabited areas that caused water, soil and air contamination. This intoxication severely affected human health, leading to renal injuries, immune deficiencies, apathies, bone injuries and pain as well as skeleton deformations: all these features provoked great lamentations thus the disease was called *Itai-Itai* (86).

Cadmium poising is mainly due to the low excretion rate of this metal, that has an half-life of about 15-20 years (87): this causes its accumulation in human body, principally targeting liver and kidneys that can contain about the 75% of the total cadmium amount (88).

While acute intoxication is related to injuries to testes, livers and lungs (86,89), chronic exposure starts to affect the airway with obstructive diseases and emphysema and then causes renal failures, diabetic and renal complications, bone disorders and immune suppression (84,90,91). Besides direct alterations of a variety of systems and organs, cadmium intoxications has been also associated to cancer development, particularly to lung cancers despite somewhat weaker correlations have been demonstrated with cancers of prostate and kidney (92) and controversial studies indicate a possible occurrence also in liver, pancreas and stomach tumors (93,94). Therefore, the International Agency for Research on Cancer (IARC) has classified cadmium as a carcinogen of category I (84), despite interestingly it is not directly genotoxicant, but it shows co-mutagen effects in combination with other mutagenic agents like UV-light in mammalian cells (95,96).

By the way, regardless the type of tissue targeted, depending on the cadmium doses used the cellular physiology is affected at multiple levels, altering cell cycle progression, proliferation, DNA replication and repair as well as triggering cellular death pathways (97–100). Notably, DNA synthesis and cell proliferation are promoted by low doses because of the activation of particular cellular responses (101–106) potentially supporting the involvement of cadmium in the onset of tumoral transformation. These responses include the promotion of RAS signaling, the

activation of protoncogenes, such as C-FOS, C-MYC and C-JUN (Table 1) (107,108) and the regulation of transcriptional and translational factors (Table 2).

Genes or gene products	System	Effective cadmium concentration or dose	Reference
Immediate early response genes and *p53*			
c-fos, c-myc	Rat myoblasts	5–10 μM	Jin and Ringertz, 1990
c-fos, nur 77, egr-1	Mouse 3T3 cells	2 μM	Epner and Hershman, 1991
c-fos, c-jun, c-myc	NRK cells	1 μM	Tang and Enger, 1993
c-fos, c-jun, c-myc, egr-1	Rat LLC-PK1 cells	1–20 μM	Matsuoka and Call, 1995
c-jun, c-myc	Rat myoblasts	0.5 μM	Abshire et al., 1996b
c-fos	Rat mesangial cells	1–10 μM	Wang and Templeton, 1998
c-jun, c-myc	Human prostate epithelial cells	10 μM	Achanzar et al., 2000
c-fos, c-jun, c-myc	BALB/c-3T3 cells	20 μM	Joseph et al., 2001
p53	Human prostate epithelial cells	10 μM	Achanzar et al., 2000
c-jun	Mouse liver	20–40 μmol/kg s.c.	Zheng et al., 1996
p53	Mouse liver	5–20 μmol/kg s.c.	Zheng et al., 1996

Table 1. ***Protoncogenes expression activated by cadmium treatments.***

The table reports the name of protoncogenes induced by the indicated concentrations of cadmium in the reported cellular system or animals (from Waisberg et al, *Toxicology* 2003).

Although these cadmium effects promote the development of tumors supporting its role in these pathologies, other reports evidence its great toxicity at higher doses due to the activation of cell death pathways as necrosis and apoptosis (109–114). Notably, treatment with high concentrations of cadmium generates a big and sudden overload of free radicals promoting a faster activation of death mechanisms (113).

Given the association of oxidative stress and neurodegeneration, recent evidences supported an important role of environmental pollutants, included cadmium, in the onset of neuronal alterations and death potentially causing the development of neurodegenerative diseases (30,104,115). Notably, cadmium uptake through inhalation from the nasal mucosa causes its accumulation into the brain both toward olfactory neurons transport (30) and also affecting the integrity and permeability of the vascular endothelium and mitochondrial membranes (116). Furthermore, the competitive role of cadmium respect of calcium ions impairs all the pathways requiring its influx, included the release of neurotransmitters, both through the block of calcium channels and also through the interaction with thiol groups of calcium binding proteins (116).

Modulation of transcription factors and translation factors by cadmium in vitro

Factor	Direction of change	System	Effective cadmium concentration (μM)	Reference
HSF1	↑	CHO cells	25	Liu et al., 1995
USF	↑	Hepa-1 cells	7	Li et al., 1998
NRF2	↑	MCF-7 cells	10	Alam et al., 2000
MTF1	↑	MCF-7 Cells	10	Smirnova et al., 2000
NF-κB	↑	Mouse macrophages	1	Misra et al., 2002
TIF3	↑	BALB/c-3T3 cells (transformed)	6–12	Joseph et al., 2002a
TEF-1δ	↑	BALB/c-3T3 cells (transformed)	6–12	Joseph et al., 2002b
HIF-1	↓	Hep3B hepatoma cells	0.5–10	Obara et al., 2003
Sp1	↓	Rat alveolar epithelial cells	5–20	Watkin et al., 2003

Table 2. ***Transcriptional factors cadmium regulated.***

The name and the dysregulation of transcriptional factors, the cellular type and the concentration of cadmium inducing these modulations are showed in the table (from Waisberg et al, *Toxicology* 2003).

In this context, the altered permeability of mitochondrial membrane contributes to increase the overload of cytosolic calcium releasing it with cytochrome c, thus sustaining the apoptotic pathway thanks to the activation of the caspase proteolytic cascade (Fig. 6). Because of the large amount of mitochondria presents within neuronal cells, this mechanism of death induced by cadmium could be mainly responsible of neurodegenerative processes related to this environmental pollutant. At any rate, in order to counteract the toxicity of cadmium, cells increase the expression levels of metallothioneins (MT) representing the main cellular mechanism of metals detoxification. Notably, thanks to the content of about 30% of cysteine residues, these proteins can both chelate cadmium and also reduce the oxidative stress induced by its presence (117). The alteration of the intracellular redox homeostasis upon cadmium administration is considered the main cause of its cytotoxicity: notably, the detection of higher amounts of superoxide anion ($O_2\cdot$) and H_2O_2 upon cadmium treatment confirm the induction of an increase of intracellular ROS species (117).

Nevertheless, although cadmium itself is not a redox-active metal and the mechanisms leading to the ROS enhancement remain largely unknown, this drug is known to induce indirect modifications of the intracellular systems devoted to the maintenance of the redox homeostasis, partially explaining the establishment of a great oxidative stress condition.

By the way, different works report an unbalance of antioxidant defenses due to a dysregulation of both levels and activity of almost of cellular enzymes and proteins required to ensure the redox homeostasis (Table 3).

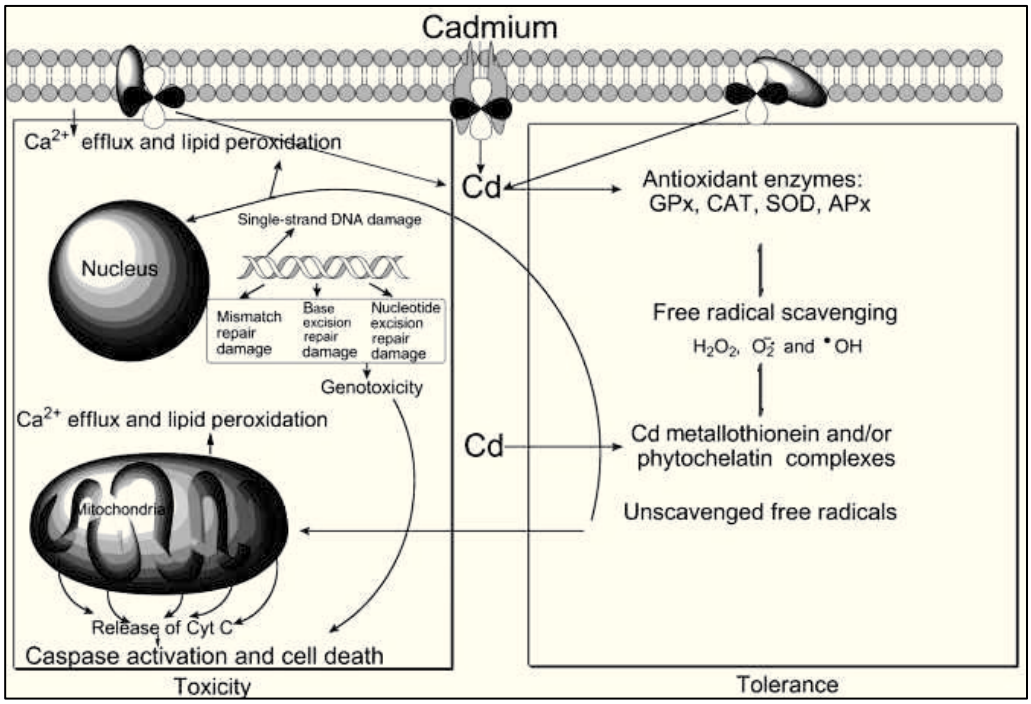

Figure 6. ***Cartoon of cadmium mechanisms triggered to induce neurotoxicity and genotoxicity in cell.***

Cadmium ions enter cells toward channels or transport proteins. Within cells it alters the activity of antioxidant enzymes (as metallothioneins). The consequent excess of free radicals increases the levels of lipid peroxidation and modifies biological membranes permeability, included mitochondrial membrane. This causes a release of calcium and cytochrome c from mitochondria into the cytosol that promote the activation of cell death pathways, notably apoptosis and/or necrosis (Image from Shanker, *Trace Elements as Contaminants and Nutrients: Consequences in Ecosystems and Human Health* 2008).

Moreover, other studies link the increased oxidative stress condition to mitochondrial alterations, and specifically to cadmium interference, with the electron transport chain complexes II and III, main responsible for ROS production (97,118). In any case, these mechanisms generate an accumulation of oxidant molecules that promotes the damage of several biological macromolecules, causing protein misfolding, lipids peroxidation and DNA alterations included bases modifications, single and double strand breaks and sister chromatin exchange (119).

In this context, the main lesion occurring in genome sequence should be represented by base oxidation damages, that are recognized and removed efficiently through recruitment of BER pathway.

Nevertheless, cadmium enhances its toxicity by upgrading the amount of DNA damages through the induction of a general dysregulation of almost all the DNA repair systems, included BER, NER and MMR. Specifically, cadmium interferes with the DNA binding activity of many repair proteins (120–125), principally targeting the

zinc finger motifs: thanks to its similarities to zinc atom, cadmium replaces this element and disrupts the finger motifs interacting with cysteine residues.

Synopsis of cadmium effects on the activity of enzymes of the antioxidant system

Enzyme[a]	Exposure duration	Effects on activity[b]	Model	Cd dose or concentration	Reference
Catalase	2 h	–	V79 cells	5×10^{-5} M	Ochi et al., 1987
	4 h	↓	CHO-K1 cells	4 μM	Yang et al., 1996
	3 h to 10 days	↑↓	Rats	0.4 mg/kg	Sarkar et al., 1998
	45 days	↓	Rats	0.4 mg/kg	Shukla et al., 1989
	30 days	↓	Rats	0.5 mg/kg	Salovsky et al., 1992
	6 h to 30 days	↓	Rats	2.5 mg/kg	Casalino et al., 2002
	30–90 days	↑↓	Rats	10 mg/kg	Shukla et al., 1996
	30 days	↑	Rats	15.0 mg/kg	Kostic et al., 1993
SOD	2 h	–	V79 cells	5×10^{-5} M	Ochi et al., 1987
	3 h to 10 days	↑↓	Rats	0.4 mg/kg	Sarkar et al., 1998
	30 days	↓	Rats	0.5 mg/kg	Salovsky et al., 1992
	30–90 days	↑↓	Rats	10 mg/kg	Shukla et al., 1996
	30 days	↑	Rats	15 mg/kg	Kostic et al., 1993
Cu/Zn SOD	6 h to 30 days	↑↓	Rats	2.5 mg/kg	Casalino et al., 2002
Mn SOD	6 h to 30 days	↓	Rats	2.5 mg/kg	Casalino et al., 2002
GSSG-R	2 h	–	V79 cells	5×10^{-5} M	Ochi et al., 1987
	24 h	↓	Pneumocyte II	1 μM	Tatrai et al., 2001
	4 h	↓	CHO-K1 cells	4 μM	Yang et al., 1996
	30–90 days	↓	Rats	10 mg/kg	Shukla et al., 1996
	30 days	↑	Rats	15 mg/kg	Kostic et al., 1993
GSH-Px	24 h	↓	Pneumocyte II	10^{-5} M	Tatrai et al., 2001
	2 h	–	V79 cells	5×10^{-5}M	Ochi et al., 1987
	4 h	↓	CHO-K1 cells	4 μM	Yang et al., 1996
	45 days	↓	Rats	0.4 mg/kg	Shukla et al., 1989
	30 days	↑	Rats	0.5 mg/kg	Salovsky et al., 1992
	30–90 days	↑↓	Rats	10 mg/kg	Shukla et al., 1996
	30 days	↑	Rats	15 mg/kg	Kostic et al., 1993
GST	3 h to 10 days	↑	Rats	0.4 mg/kg	Sarkar et al., 1998

Table 3. ***Effect of cadmium on antioxidant defenses.***

The table summarizes the result of cadmium administration on the activity of different antioxidant systems, indicating doses and times of treatments and reporting the cellular system or the animals used for the experiments (from Waisberg et al, *Toxicology* 2003).

To support the involvement of this feature of cadmium in mediate the alteration of DNA repair proteins, several reports demonstrated a recovery of binding activity of a lot of them upon addition of zinc to cadmium treatment (120,124,125). Furthermore, besides this, cadmium can impair also the repair activity of several proteins directly replacing metal ions (mainly Mg^{++} or Mn^{++}) essential to improve both coordination of key amino acid residues within the active site and the efficiency of their same DNA repair activity.

Despite these mechanisms can damage the efficacy of all repair pathways, the effect of cadmium on BER mechanism and its proteins remains the most studied because of the potential key role of this pathway to counteract and repair DNA oxidative lesions cadmium induced.

Notably, OGG1 and Ape1, the first two proteins recruited in BER mechanism, result mainly affects by this metal. In particular, different studies have reported a dysregulation both of the expression levels and of the activity of OGG1 (126–128):

these two effects could be respectively due to a transcriptional down-regulation, caused by a reduced binding activity of the transcriptional factor *Sp1* and to the inactivation and sequestration of the same DNA glycosylase into stress granules (127). Otherwise, cadmium treatment affects the activity of Ape1 *in vitro* (26) but not *in vivo* (127), even if other reports suggest that this alteration could be related to *p53* state, directly involved in the modulation of Ape1 expression, thus contributing to the alteration of its activity (126). Nevertheless, although cadmium affects also the activity of poly(ADP-ribosylation) protein (PARP), an important accessory protein essential for BER pathway coordination (122), no data are available regarding cadmium effects on the other BER enzymes directly employed to solve the oxidative lesions.

To sum up, cadmium toxicity is mainly caused by both the alterations of cellular antioxidant defenses and the impairment of DNA repair systems: this condition promotes the accumulation of free radicals with a consequent increase of genome instability (Fig. 6), thus leading to the activation of proliferative mechanisms or cell death pathways.

In particular, the increasing evidences of a role of this pollutant in the onset of neurodegenerative processes point out the necessity to better define how its toxicity can be counteracted. Therefore, it is of great interest to better define the effects of cadmium on BER proteins levels and to develop a strategy in order to modulate its toxicity on neuronal cells.

Cisplatin as crosslinking agent

Cisplatin (cis-Diamminedichloroplatinum(II)) is a drug widely used for the treatment of several types of cancer. As many other chemotherapeutic agents, it targets genome by inhibiting both DNA replication and transcription thus leading, as a final result, to the induction of cancer regression through the activation of apoptotic pathways (129). Cisplatin belongs to the family of platinum derived molecules, together with the cis-Diammine-1,1-cyclobutane dicarboxylate and the (trans-R,R)1,2-Diaminocyclo-hexaneoxalatoplatinum(II), respectively carbo- and oxaliplatin. Although they have some similarities about the structure (Fig. 7), some relevant differences relative to pharmacokinetics and adverse effects address to a specific therapeutic use of each of them (130–133).

Cisplatin was the first drug derived from platinum and, in 1978, it started to be used mainly for the treatment of testicular and ovarian cancers (134). Nevertheless,

although its high efficacy, the induction of a considerable cytotoxicity also on normal tissues prompted to look for other less toxic analogues . Therefore, it was developed a second-generation of platinum drugs, included carboplatin. Despite it is equally effective respect to cisplatin for the therapy of some cancer types, carboplatin is usually combined with other drugs mainly for the treatment of highly aggressive ovarian and lung cancers (135–138). At any rate, both cisplatin and carboplatin are significantly cytotoxic and mutagenic, as observed in cell culture and animal models (139,140). In addition, during these chemotherapeutic treatments, many tumors point out the onset of resistant phenomena derived by both cancer intrinsic and therapy-induced features (21,141,142).

In order to reduce these adverse effects, it was worked out a further generation of platinum derived molecules: oxaliplatin belongs to this third group and it is usually employed, alone or in combination with other drugs, for the therapy of metastatic cancers of colon and rectum (143,144). Notably, it is less mutagenic than cisplatin and carboplatin and appears often effective against tumors resistant to the other platinum derivatives (145–147).

Although all platinum compounds act as crosslinking agents binding the same types of bases at the same sites on DNA (148–150), it has been demonstrated that their cytotoxicity is strictly related to the recognition of DNA adducts by specific DNA repair systems primarily dependent on the distortion of genome structure (151). Specifically, while cisplatin and carboplatin adducts share a similar conformation exploiting the same cis-diammine carrier ligands to bind DNA, oxaliplatin uses a (trans-R,R)1,2-diaminocyclohexane radical (Fig. 7) inducing a different bending.

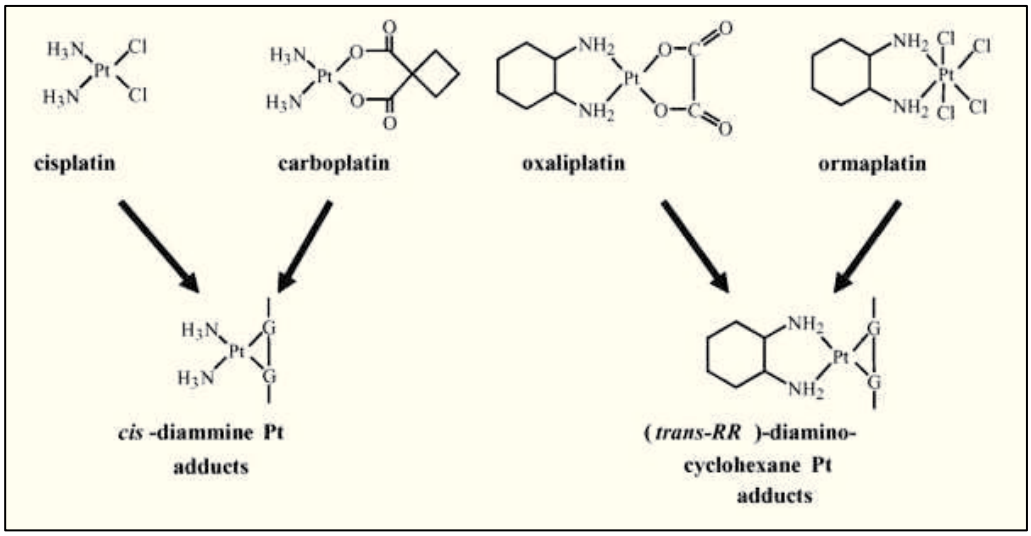

Figure 7: ***Platinum compounds.***

The figure shows the structure of three platinum derivatives with the relative DNA adducts (Image adapted from Chaney et al, *Critical Reviews in Oncology/Hematology* 2005).

Nevertheless, regardless the platinum derivatives used, the ratio among the types of DNA adducts formed is approximately unchanged: only about 1-3% of them may arise between two complementary DNA regions (interstrand crosslinks, ICLs) usually involving two guanines (GG adduct), while the most of platinum lesions are located in the same strand, intrastrand adducts, and can be formed by different bases, although GG adducts are more common (60-65%) than AG (25-30%) and GNG (5-10%) lesions (152,153).

Several DNA repair pathways are involved in the removal of these types of lesions, as supported by the recruitment to the site of platinum-adducts of many components belonging to both NER and HR as well as trans-lesion DNA synthesis pathways (23,154–156). Notably, while intrastrand lesions are mainly repaired through NER mechanism (157), ICLs appear require both NER and HR pathways but also the activation of trans-lesion DNA synthesis mechanisms (158,159) that allows the replication of damaged sequence without repair of the lesion. Therefore, although the binding of platinum compounds differently bend and distort DNA structure, many studies underline that some of these pathways, as NER (148,157) and HR (23) probably share a similar mechanism of recognition regardless of the platinum drugs. This is supported also by the similar dysregulation of cell sensitivity induced by any defect of enzymes involved in these mechanisms (160–162). In contrast, proteins of trans-lesion synthesis, in particular polymerases, are able to discriminate between the two different adduct conformations. Although several polymerases can be

recruited in this mechanism (163–165), the most employed are DNA polymerase μ and β (151). Nevertheless the involvement of Polβ in this mechanism is mainly associated with its overexpression (166,167) but both the enzymes show an higher ability to bypass lesions oxaliplatin-induced rather than those cisplatin related (168,165). This aspect could be explained by the specific folding of the cisplatin-DNA adduct that hinders the binding site of the DNA polymerase (169). However, this unequal processing could support the lower mutagenic profile of oxaliplatin (151), thanks to the relative error-free DNA synthesis carries on by polymerase μ to bypass platinum adducts (163).

Furthermore, it is thought that this different adducts recognition could potentially explain the effectiveness of oxaliplatin treatments on cisplatin resistant cells, as well as the different cytotoxic profile of these drugs (151). By the way, other DNA repair pathways, as MMR and BER, appear to promote cellular sensitivity specifically to cisplatin treatment (22,170–173). Notably, although the MMR proteins enhances its cytotoxicity (174–176), recent evidences demonstrated a strict dependence between BER recruitment and MMR activation in order to mediate a sensitizing effect mainly to cisplatin interstrand adducts (177).

In particular, ICLs generate a significant distortion of DNA double helix that causes a flipping out of adjacent cytosines (178), resulting more prone to deamination events (173). This leads to a prompt recruitment of BER proteins, that are not able to completely restore the correct sequence (177).

In fact, previous works pointed out that the incorporation of correct nucleotides past adducts was related to DNA sequence (168,164) and, recently, it has been observed an high rate of Polβ nucleotide misincorporation upon cytosine deamination (177). Although this aspect could explain the mutagenic profile of cisplatin, the generation of mismatch points (173) through Polβ synthesis induces the recruitment of MMR proteins, leading to an increased cisplatin cytotoxicity (177). Notably, the key role of Polβ is essential to ensure an increased cisplatin cytotoxicity through this mechanism, as supported by the onset of cisplatin resistance phenomena upon dysregulation of either expression or activity of this specific enzyme (173,179,180). Thus, starting from these evidences, it has been recently proposed that BER and MMR proteins recruitment, upon deamination of cytosine flanking cisplatin ICLs, could avoid the processing of adducts by NER pathway, thus promoting a non-productive repair of the lesions. Therefore, as a final result, the persistence of DNA adducts may increase cisplatin cytotoxicity (177).

Nevertheless, although it is expected that cisplatin toxicity has to be mainly appreciated in cycling cells, the induction of the only DNA damages hardly explains the cytotoxicity exerted also on normal post-mitotic tissues.

By the way, peripheral neurotoxicity has been observed as a side effect upon treatment with all these platinum compounds. In particular, although carboplatin treatment is less toxic, cisplatin and oxaliplatin toxicity have a similar side effect (181–183) resulting in the induction of the same significant neurological dysfunctions (Table 4).

Different hypothesis have been proposed to support the onset of this adverse effect, although the most reliable involves the induction of oxidative stress condition. Notably, while the addition of antioxidant molecules to the treatment has been associated to a reduced toxicity of the drugs (184–186), increased levels of ROS species have been detected upon cisplatin exposure in different tissues (187–189).

Comparison of cisplatin- and oxaliplatin-induced neurotoxicity	Cisplatin	Oxaliplatin
Dose limiting toxicity	Peripheral neurotoxicity	Peripheral neurotoxicity
Symptoms	Paresthesia	Paresthesia, sensory ataxia, and dysesthesia
Location	Extremities	Extremities, perioral area
Time-course onset	Delayed	Acute and delayed
After treatment	deterioration	Recovery
Accompanying toxicities	Ototoxicity	Laryngospasms
Precipitating factors	None	Exposure to cold

Table 4. ***Neurological alterations induced by platinum compounds.***

Comparison between neurotoxicity induced by cisplatin or oxaliplatin treatments (Image from Amptoulach et al, *Chemotherapy Research and Practice* 2011).

Furthermore, recent data demonstrated that mitochondria, representing the most important endogenous source of ROS, are directly involved in mediate cisplatin cytotoxicity, as supported also by the reduced sensitivity observed after depletion of the mitochondrial genome (190–192). In particular, mitochondrial dysfunctions, included both DNA and proteins adducts (189,193), as well as impairment of respiratory chain, are caused by cisplatin accumulation and lead to the activation of cell death pathways, like apoptosis that is the most observed upon cisplatin treatment (194–196). Specifically, the impairment of the respiratory mitochondrial chain is the direct responsible for the generation of oxidative stress conditions (197,198). As further proof of the key role of mitochondria, it has been demonstrated that sensitivity to cisplatin treatment results mostly enhanced in cells containing a large amount of them, that is strictly related to an higher tendency to produce ROS upon exposure to damaging agents (192). This last evidence may potentially explain the great

cytotoxicity of cisplatin on neuronal cells: in fact, the oxidative stress induced by the drug could further increase the basal levels of ROS species physiologically produced by endogenous sources, as both the high metabolic rate and the great content of mitochondria, characterizing this type of cells (187). It is known that an overload of ROS radicals promotes the onset of oxidative DNA damages, therefore this condition could possibly enhance cisplatin toxicity. Moreover, in this context, it could be hypothesized a possible involvement of BER pathway in mediating cisplatin neurotoxicity. By the way, some reports demonstrated an enhanced neuronal sensitivity upon impairment of BER mechanism supported also by the increase of specific BER proteins following cisplatin exposure (199–201).

In summary, to date, BER pathway appears clearly involved in modulating cisplatin sensitivity. Notably, while its recruitment in cycling cells could enhance the cytotoxic effect of this drug (173,177), on the other hand this mechanism is essential in modulating the neurotoxicity deriving from cisplatin exposure. By the way, further analysis could better define the effectiveness of BER proteins in mediate protection of nervous system, possibly delineating, also in this mechanism, a leading role of a specific BER protein, as observed for Polβ in the modulation of cisplatin toxicity.

2.2.3 BER proteins

BER pathway leads to the restoration of the correct DNA sequence following the removal of lesions specifically occurring on nucleotide bases, thanks to the activity of specific proteins recruited following a well-defined order.

DNA Glycosylases

DNA glycosylases (DG) act in the first step of BER mechanism, directly recognizing the damaged bases. Although all proteins belonging to this class of enzymes share the same glycosylase activity, they are able to discriminate among the different damaged bases. By the way, eleven mammalian DG have been characterized and are mainly divided in two subclasses, depending on the type of activities owned (Table 5). Thus, while monofunctional or “pure glycosylases” have only a glycosylase activity, on the other hand bifunctional enzymes show also an AP-lyase function, permitting to bypass the recruitment of AP endonucleases in order to cut the DNA backbone. In fact, the only glycosylase activity of these enzymes induces a specific recognition and removal of the damaged bases by a “flipping out” mechanism, thus

leading to the formation of an apurinic/apyrimidinic site (AP) on the DNA sequence without affecting the sugar phosphate backbone.

Specifically, the removal of the lesion is strictly related to the specific joint between the enzyme active site and the damaged base. Analysis of the base excision mechanism revealed that DG bind the minor groove of DNA, bending it in correspondence with the lesion, and then push the damaged base out of the major groove. Following this flipping out, only damaged bases correctly accommodated into the DG active site pocket are efficiently removed. The catalytic cleavage of the N-glycosidic bond occurs on the same anomeric carbon of the substrate, although the nucleophile involved in the reaction depends on the type of glycosylases. Notably, while monofunctional enzymes exploit an activated molecule of water, bifunctional glycosylases use an amine residue for the nucleophilic attack (202).

Glycosylase	Enzyme	Substrate	Type
UNGs	UNG	U	Monofunctional
	TDG	U:G, T:G	
	SMUG1	U, OHmeU	
HhH	MBD4	U:G and T:G in CpG sites	Monofunctional
	OGG1	8-oxoG:C, faPyA, faPyG	Bifunctional
	MYH	A:8oxoG	Monofunctional
	NTH1	TG, DHU, faPy	Bifunctional
H2TH	NEIL1	faPyA, faPyG, DHU, TG, 8-oxoG	Bifunctional
	NEIL2	5-OHU, DHU	
	NEIL3	Unknown	Bifunctional?
AAG	AAG	3-meA, 7-meG, hypoxanthine	Monofunctional

Table 5: ***DNA glycosylases features.***

The table report the different enzymes belonging to each class of glycosylases, showing the substrates recognized and the functions owned (Image from Vascotto and Fishel, *Academic Press* 2012).

Among human enzymes belonging to this class, 8-oxoguanine DNA glycosylase (OGG1) is the most studied one. Human OGG1 gene shows an high homology to the Saccharomyces cerevisiae one (203) and encodes for a bifunctional glycosylases specifically cleaving the N-glycosidic bond between deoxyribose sugar and the damaged base (204,205). Although the main substrate of this enzyme is the 8-oxoguanine, which is named OGG1, some studies demonstrated that this proteins may also remove bases with other damages, as 2,6-diamino-4-hydroxy-5-

formamidopyrimidine (FapyG) (206) and 7,8-dihydro-8-oxoadenine (8-oxoA) (207,208).

AP endonucleases

DNA AP-sites are promptly processed by enzymes harboring a specific AP endonuclease activity. Different mechanisms have been identified leading to the loss of DNA bases. In fact, although these events can arise spontaneously, AP-sites formation can be induced by both free radicals and alkylating agents that destabilize the N-glycosylic bond causing the release of the bases, as well as by DNA glycosylase recruited in BER pathway in order to remove damaged bases (36). Notably, AP sites deriving from BER processing are normally cleaved by the major human AP endonucleases an homolog of the bacterial Exonuclease III, known as Ape1 protein (Apurynic/apyrimidinic endonuclease1) (209). Interestingly, several evidences point out the essential role of this enzyme in cellular physiology, which is supported by the lethal phenotype observed in embryonic nullizygous mice and by the fruitless attempts to create stable Ape1-knockout cell lines (210). As a further proof, Ape1-knockdown in human cells causes AP sites accumulation resulting in an impaired cell proliferation and activation of apoptotic death pathways (211). Ape1 endonuclease activity is enhanced by Mg^{++} that, located in the active site, coordinates the cutting of the DNA backbone in correspondence of the AP site. Notably, cleaving the 5' phosphodiester bond, Ape1 catalytic activity creates a gap with an hydroxyl group and a deoxyribose phosphate (dRP) to the 3' and 5' ends, respectively (212). Nevertheless, although it plays a key role in BER pathway, other important functions can be ascribed to this protein (24), such as the redox activation of several transcriptional factors and the involvement in RNA metabolism.

DNA polymerases

Following Ape1 cleavage of the AP site, the generation of a 3'-hydroxyl end represents the correct primer for the DNA synthesis thus promoting the recruitment of DNA polymerases. Nevertheless, to efficiently complete the repair of the damaged sequence, the 5'-dRP terminus has to be cut out allowing the final reaction step, catalyzed by a DNA ligase (213). Depending on the mechanisms used to remove the 5' residue, two BER sub-pathways can be distinguished: the *short-patch* (SP) and the *long-patch* (LP), employing different enzymes, respectively (53).

In detail, while SP exploits DNA polymerases that remove of the 5'-dRP end through their dRP lyase activity replacing only the damaged base, on the other hand in LP the elimination of the 5'-dRP residue is associated to the displacement of further nucleotides (53).

In particular, although different DNA polymerases own the ability to remove the blocking 5'-dRP residue through a β-elimination reaction thus exploiting the dRP lyase activity, DNA polymerase β is the enzyme preferentially recruited in SP pathway. Notably, the incorporation of nucleotides is ascribed to the C-terminal domain containing the active site responsible for polymerization activity, while the dRP lyase function is located in the N-terminal (214,215). Nevertheless, as polymerase β lacks of the proof-reading activity, it has been calculated that, in mammalian cells, during repair through SP pathway the frequency of nucleotide mis-incorporation is about $5\text{-}10x10^{-4}$. Therefore, given the role of this enzyme both in BER pathway and in the maintenance of genome integrity, overexpression of polymerase β has been observed in different type of cancers while its impairment is related to apoptosis, chromosomal breaking and enhanced sensitivity to alkylating agents (216,217) as well as to the recent observed onset of specific neurodegenerative phenotypes (218).

However, although polymerase β is mainly employed in SP, it has been reported that also the processing through LP is initiated by the same enzyme: in fact, when the 5'-dRP residue appears resistant to the removal towards the dRP lyase activity of polymerase β, other DNA polymerases are promptly recruited to bypass this terminus continuing the strand synthesis through the LP pathway (51). This mechanism exploits DNA polymerase δ and ε that remove and replace other nucleotides in addition to the damaged base thus creating a flap of 2-8 bases following removed by FEN-1 (45). Furthermore, the employment of these enzymes also in DNA replication ensures the incorporation of nucleotides in an high-fidelity manner while the recruitment of PCNA, another component of replicative machinery, promotes the high processivity of the repair through LP pathway (52,53).

DNA ligases

As mentioned before, to definitively restore the correct sequence, sealing between the 3'OH and the 5'P termini of the nick leaved by polymerases is necessary . This step is performed by a DNA ligase exploiting the energy derived from the hydrolysis

of a phosphoanhydridic bond of an ATP molecule to form a phosphodiester one (219). Depending on the type of BER sub-pathway initiated, the DNA ligases (Lig) employed can be respectively LigI (DNA ligase I) mainly for LP and LigIII (DNA ligase III) preferentially in SP.

Although LigI belongs to the replicative machinery, ensuring the joint of Okazaki fragments (220), several data underline its crucial role in BER pathway, as supported by the high sensitivity detected in cells impaired for LigI activity upon exposure to damaging agents, as ionizing radiation and alkylating compounds (201–203).

Regarding LigIII, between the two identified isoforms α and β, DNA LigIIIα is the main employed in SP pathway as demonstrated by the interaction with the scaffold protein XRCC1 with which was originally purified (57,221).

BER "auxiliary" proteins

Although all the enzymes described above can restore the correct sequence of DNA upon removal of the damaged base *in vitro*, other additional proteins, as XRCC1 and PARPs, are required for the *in vivo* BER processing. As they do not show any specific enzymatic activity, it has been suggested an involvement in BER pathway as scaffold proteins providing a manner to coordinate the recruitment of the other essential enzymes.

Notably, the relevance of XRCC1 in cell physiology is pointed out by the embryonic lethality induced upon its gene knock out (222), thus supporting the crucial role of its recruitment in correspondence of the AP site in the BER mechanism. Furthermore, the physical interactions observed with other BER proteins, as DNA glycosylases, Ape1, Polβ and LigIIIα, confirms is role of scaffold protein (55,57,221).

On the other hand, PARPs proteins belong to a family of regulatory proteins involved in various cellular processes. In particular PARP-1 and PARP-2 acting as sensor of single strand break are the components involved in BER pathway. Although their role is not completely understood, PARP-1 could potentially promote the access of BER enzymes to the site of damage modifying the histone through poly(ADP)-rybosilation or interact with XRCC1 and Polβ furthering their recruitment (223).

2.3 BER and diseases

Considering the crucial role played by the BER pathway in the maintenance of genome integrity and stability, it is expected that impaired expression and/or activity of its components can potentially be involved in the onset of various human diseases. Notably, many reports have etiologically associated the presence of persistent oxidative damages, or their defective or inefficient repair, to the development of cancer and inherited or acquired neurological disorders, as well as in aging (12,14).

2.3.1 BER involvement in cancer

The development of tumors appears as a multistep process, where specific mutations induce the onset of malignant phenotypes characteristic of cancer cells and responsible for their clonal expansion. Nevertheless, several molecules can potentially modulate or activate this mechanism, included those regulating oxidative stress conditions (205).

By the way, it has been largely demonstrated that oxidative mechanisms are implicated in the initiation, promotion and progression of carcinogenesis, as well as in many other pathological conditions (Table 6).

Notably, high levels of ROS contribute to the induction of carcinogenesis processes causing both altered gene expression patterns and increased oxidative DNA damages. Furthermore, specific features of tumor microenvironment may promote the establishment of oxidative stress conditions, as the impairment of antioxidant defenses and the basal induction of ROS, like H_2O_2, without specific exogenous stimulation (12,13). In addition, it has been recently demonstrated that cancer susceptibility is increased by various single nucleotide polymorphisms (SNPs) within genes codifying for both oxidative DNA repair and antioxidant enzymes (11,224).

In fact, it is known that the polymorphic variants of antioxidant genes can differently affect the enzymatic activity of the relative proteins, resulting in a variable protection against oxidative stress. By the way, specific SNPs of some antioxidant enzymes as superoxide dismutase 1 (SOD1) and glutathione peroxidases (GPX) have been associated with an elevated risk to develop breast, lung, ovarian, prostate and bladder cancer (224–229).

Starting from the relevance of these associations, several studies have analyzed also the relationship existing between cancer development and DNA repair systems, specifically evaluating the frequency of SNPs within genes of repair proteins (11).

Unexpectedly, these analysis pointed out a weak and controversial association between polymorphic variants of DNA repair genes, notably BER genes, and predisposition to develop tumors (8,11).

Nevertheless, an exception can be represented by polymerase β: it has been observed that about the 30% of tumors express polymorphic variants of this protein. At least two of them exhibit a reduced fidelity in nucleotide incorporation, supporting a possible role in the onset of tumor mutations (230–232), while the others result mainly truncated or deleted showing respectively the loss of the DNA-binding domain or an impaired enzymatic activity (233,234).

Breast	Invasive ductal carcinoma	8-OH-Gua, FapyGua, 8-OH-Ade, FapyAde (DNA)	• Levels of lesions, apart from FapyAde, significantly ($P \leq 0.01$, $P \leq 0.02$, and $P \leq 0.05$, respectively) increased compared to calf thymus DNA (116).
	Breast cancer	8-OH-dG (DNA)	• Levels of 8-OH-dG were not significantly elevated in breast cancer tissue vs control, nor were levels associated with expression of oestrogen/progesterone receptors, clinical stage, or histological grade (117).
	Invasive ductal carcinoma	8-OH-dG (DNA)	• Significantly elevated levels of 8-OH-dG ($P < 0.001$) in malignant breast tissue; also levels significantly greater ($P = 0.007$) in estrogen receptor-positive (ORP) vs. ORP-negative malignant tissue (118).
	Primary breast cancer	8-OH-dG (DNA)	• Significantly higher ($P < 0.0001$) levels of 8-OH-dG in tumour vs. nontumor tissue (119).
Cardiovascular disease		8-OH-dG (DNA)	• Strong association ($r = 0.95$, $P < 0.01$) between premature coronary heart disease in men and lymphocyte 8-OH-dG levels (120).
Colon	Colorectal cancer (CRC)	8-OH-dG (DNA)	• Significantly elevated levels of 8-OH-dG ($P < 0.005$) in tumor tissue compared to normal mucosa (121).
Colon (*continued*)		8-OH-dG (DNA)	• Significant correlation between lymphocyte 8-OH-dG levels and colorectal cancer deaths in men ($r = 0.91$, $P < 0.05$, ref. 120).
Gynaecological	Gynecological cancers	8-OH-dG (urine)	• Levels significantly higher ($P \leq 0.05$) in patients with gynecological cancer compared to control subjects.[b]
	Cervical cancer	8-OH-dG (DNA)	• Levels of 8-OH-dG significantly increased ($P < 0.001$) in low- and high-grade levels of dysplasia, compared to normal, although this did not correlate with human papillomavirus status (122).
Kidney	Renal cell carcinoma (RCC)	8-OH-dG (DNA)	• Levels of 8-OH-dG significantly higher ($P < 0.0005$) in RCC vs. noncancerous tissue (123).
	Transplantation	dTg (urine)	• Significantly elevated levels of dTG after kidney transplantation proposed to be due to ischemia-reperfusion injury (124).
Liver	Haemochromotosis	8-OH-dG (urine)	• No significant difference in levels between patients and control subjects.[b]
	Wilson's disease and primary hemochromotosis	8-OH-dG (DNA)	• 8-OH-dG levels not elevated in liver of hemochromotosis patients and significantly lowered in liver of Wilson's disease (125).
	Chronic hepatitis	8-OH-dG (DNA)	• Liver levels of 8-OH-dG significantly elevated ($P < 0.05$) compared to controls (126).
	HCV	8-OH-dG (DNA)	• Leukocyte DNA levels 8-OH-dG significantly higher than in HBV infection ($P < 0.04$), correlating with clinical diagnosis ($P < 0.025$) (127).
	HCV	8-OH-dG (DNA)	• Significantly elevated levels of liver 8-OH-dG compared to controls ($P < 0.001$; 128).
	HCV	8-OH-dG (DNA)	• PBMC levels of 8-OH-dG significantly elevated ($P < 0.00001$) in HCV-positive patients, compared to controls. 8-OH-dG levels positively correlated ($P < 0.02$) with presence and extent of liver damage (129).
	Hepatoblastoma	8-OH-dG (DNA)	• Positive immunohistochemical staining for 8-OH-dG in liver sections from all 5 patients with hepatoblastoma (130).
	Chronic hepatitis, alcoholic liver disease, primary biliary cirrhosis,	8-OH-dG (DNA)	• Positive immunohistochemical staining for 8-OH-dG in all diseased liver sections; no staining in control liver sections (131).
	Hepatocellular carcinoma (HCC)	8-OH-dG (DNA)	• Significantly ($P < 0.005$) elevated levels of 8-OH-dG in peritumoural tissue compared to tumor tissue in HCC. In contrast, patients with hepatic metastases (non-HCC) or end-stage alcoholic liver disease showed no differences between the corresponding two regions (132).
Lung	Cystic fibrosis	8-OH-dG (urine)	• Urinary levels of 8-OH-dG significantly raised vs. control subjects.[b]
	Squamous cell carcinoma (SCC)	8-OH-Ade	• Levels elevated in tumor tissue of all SCC patients vs. controls,
		8-OH-Gua	- levels elevated in 4/5' patients,
		FapyGua	- levels elevated in 3 patients,
		5-OHMe-Ura, 5-OH-Ura, 5-OH-Cyt, 2-OH-Ade	- levels elevated in ⅗ patients,
		5-OH-Hyd, 5,6-diOH-Ura, FapyAde (DNA)	- levels elevated in only ⅛ or ⅝ patients (133).
	Small cell carcinoma	8-OH-dG (urine)	• Elevated 8-OH-dG compared to controls ($P < 0.05$).[b]
	Non-small cell carcinoma	8-OH-dG (DNA)	• No significant differences in 8-OH-dG levels in tumour compared to nontumor tissue (84).

Table 6: ***Reports of pathologies in which oxidative damages have been measured.***

The table shows the pathologies relative to the tissue reported with the oxidative damages detected (Image adapted from Cooke et al, *The FASEB journal* 2003).

However, regarding the other BER proteins, the association between the development of specific types of cancers and polymorphic variants of one or more BER proteins has not been well defined, yet.

Notably, OGG1 mutations are rarely observed in cancer patients while, only in few cases, its SNPs result in a mild reduction of the enzymatic activity, (235–237). Therefore, although it has been proposed the existence of a possible correlation between specific OGG1 SNPs and increased risk of lung, esophageal, prostate and gastric cancer (9,238,239), conflicting reports, supported by the lack of correlation between OGG1 polymorphic variants and both pancreatic cancer or squamous cell carcinoma of head and neck (240,241), suggest that probably other factors may promote the enhanced susceptibility to disease (242).

Similarly, despite several polymorphic variants of Ape1 and XRCC1 result in an impaired activity of these proteins, a direct involvement of their SNPs in cancer development cannot be definitively established (9,10,243,244).

In particular, among the eighteen polymorphisms reported for Ape1, only some of them have been found in lung, colon, breast, head and neck, prostate and pancreatic cancer, but also in these cases there is not a strict correlation among tumor and SNPs (9,10,240,245). Nevertheless, it has been observed that Ape1 haploinsufficient mice present an high spontaneous mutation rate associated to an increased predisposition to cancer development, as well as to a reduced survival following oxidative stress exogenously induced (246). In addition, interestingly, alteration of both localization and expression level of Ape1 have been detected in several types of cancer, also at different levels of carcinogenesis process, suggesting a potential involvement of this protein in tumors development (25,27).

Nevertheless, although the polymorphic variants of BER proteins weakly and differently correlate with cancers, the embryonic lethality caused by the complete knockout of one of the core BER proteins (Ape1, Polβ, XRCC1, DNA LigI or III) suggest the essential role of these enzymes for survival (247,248). Therefore, some SNPs found in human population could potentially result in damages accumulation and genome instability possibly enhancing cancer susceptibility upon additional exposure to exogenous harmful factors (8). Recently, this mechanism is of particular interest because of the increasing evidences supporting a potential role of environmental pollutants, as heavy metals, in the development of specific types of tumors mainly inducing a dysregulation of both expression and activity of DNA repair

proteins, included BER enzymes (97,121,249), thus supporting the combined role of genetic and environment components in cancer development.

2.3.2 BER in neuronal pathologies and aging

The central nervous system (CNS), because of the high physiologic production of ROS species, is normally exposed to oxidative stress conditions. Therefore, it is not surprising to observe an higher concentration of age-related oxidative DNA damages compared to that detected in other tissues. Nevertheless, the appearance of these lesions in a specific area of CNS is usually associated with the onset of neurodegenerative diseases (250), as supported by the large amount of oxidative damages detected in neurons of patients affected by amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) (251). Furthermore, abnormal protein aggregation, typical of neurological disorders as PD, Alzheimer's disease (AD) and Huntington's disease (HD), enhances the cellular oxidative stress condition altering mitochondrial physiology, thus increasing ROS production and promoting apoptotic cell death (252).

The accumulation of oxidative damages can be mainly ascribed to an impairment of repair mechanisms age-induced, directly caused by a dysregulation of the enzymatic activity of various proteins, mainly BER enzymes (Table 7), rather than to an aging effect on the major antioxidant systems (14).

Notably, polymorphic variants of Ape1 have been correlated to an higher predisposition to ALS, as well as XRCC1 was associated to the onset of autosomal recessive spinocerebellar ataxias (253–256). Furthermore mutations and altered expression of BER proteins, as OGG1 and Ape1 but also deficiency of DNA polymerase β have been associated to an increased human predisposition to various hereditary neurodegenerative diseases, as AD (218,257).

Nevertheless, because the general impairment of BER capacity hardly correlates with an altered expression of BER proteins in brain tissues, it has been proposed that, as for carcinogenesis, the development of neurodegenerative disease is induced by a multicausal mechanism (14,258), probably involving environmental factors.

According to this hypothesis, the age-related impairment of DNA repair mechanisms allows to appreciate, only in old age, the effect of exogenous factors, to which human population is normally exposed. This is the case of some environmental pollutants,

as heavy metals (i.e. cadmium, copper, iron,…), which accumulation has been associated to the onset of several neurological disorders included AD, ALS and PD, as demonstrated by their high detection in these damaged tissues (79,259).

BER enzyme	Variation	Pathology/treatment	Brain tissue or region
Base removal			
OGG1	Increase	Forebrain ischemia and reperfusion	CNS
	Increase	Parkinson's Disease	Substantia Nigra
	Increase	Diethylmaleate	Cerebellum, cortex, and pons/medulla
	Decrease	Aging	Cerebellum and brain stem
NTH1	Decrease	Aging	Cerebellum
UDG	Increase	Ischemia	Cortex
AP site incision			
Ape1/Ref-1	Decrease	Hypoxic-ischemic injury	Hippocampus
	Decrease	Cold injury-induced brain trauma	Hippocampus
	Decrease	Transient focal and global cerebral ischemia	Hippocampus
	Decrease	Compression injury	Cortex
	Decrease	Ischemia	Spinal cord
	Increase	Seizures induced by kainic acid	Hippocampal CA1, CA3 and hilar subregions, pyriform cortex, amygdala, and thalamus
	Increase	Hyperoxia, young rats	Forebrain and hippocampus
	Decrease	Sporadic ALS	Frontal cortex
	Increase	ALS	Brain and spinal cord
Repair synthesis			
β-pol	Increase	Hypoxia	Cerebral cortex
	Decrease	Aging	Cerebral cortex
Ligation			
DNA Ligase I	Decrease	Aging	Cerebral cortex

Table 7: ***Base excision repair variations in brain aging and pathology.***

The table summarize the change observed in BER enzyme reporting the pathology and the brain region in which it has been observed (Image adapted from Fishel et al, *Mutation Research* 2007).

Normally, different mechanisms of cellular detoxification are exploited to remove these toxic metals: specific proteins, as metallothioneines or ferritin and transferrin, are involved in these processes by, respectively, chelating or sequestering heavy metals thus acting as scavenger or storage proteins. Nevertheless, the cytotoxic effect of these pollutants mainly derives by their intracellular accumulation causing an overload of the detoxification mechanisms. This is accomplished through direct interference with structure and/or activity of the involved proteins, saturation of their binding sites or dysregulation of their synthesis thus leading to the presence of free intracellular metals (30,260).

The increase of free metals amount represents a potential threat for the cells: notably, most of them may enhance the intracellular ROS production, thus promoting the establishment of oxidative stress conditions and, particularly at high doses, the activation of cell death mechanisms, including necrosis and apoptosis (100,113,118,261).

In addition, an impairment of DNA repair mechanisms, particularly BER proteins, has been observed upon neuronal exposure to several heavy metals, starting from low

non-cytotoxic concentrations of treatment (262–264). As a result, this effect, by causing a further imbalance between genome oxidative damages and repair capability of CNS, could further support the involvement of heavy metals in the onset of neurodegenerative diseases (265).

Notably, it has been demonstrated that elevated levels of iron impair the binding activity of FEN-1 and LigIII (263) and completely inhibit NEILs, a specific class of DNA glycosylases particularly expressed in CNS, thus impairing also the downstream interaction with other BER proteins, as DNA polymerase β (266). Furthermore, treatments with various metals, as cadmium, arsenic, nickel, cobalt as well as iron, may affect the DNA binding activity of several DNA repair proteins. Specifically they interfere with the zinc finger domains towards a displacement of zinc ions, thus hindering the catalysis of enzymatic reactions performed by these proteins, but also affecting the coordination of the same BER enzymes as supported by the metal interference with a scaffold protein as PARP (252,250).

A similar mechanism has been suggested to explain the direct inhibitory effect of these metals on the active site of some proteins using specific essential metals in order to catalyze their reactions. By the way, it has been observed that iron and cadmium can replace magnesium or manganese ions significantly affecting Ape1 and DNA polymerase β activity (26,263). Moreover, another potential mechanism of BER protein inactivation has been recently ascribed to this metal: notably, although OGG1 expression levels can be transcriptionally reduced by cadmium treatments (267). Further data demonstrated that exposure to this metal can further affect the intracellular level of this glycosylase towards oxidative inactivation and consequent recruitment of the inactive form of OGG1 in stress granules (127).

An additional involvement of BER proteins, notably Ape1, has been proposed in other neurological disorders, involving the peripheric nervous system (PNS) as the sensitive neuropathy induced by chemotherapeutic treatments (187,199,268). Although it is not clear the mechanism through which anticancer drugs may directly alter the physiology of sensitive peripheral neurons, oxidative stress is emerging as an important factor in mediating this effect (269).

Notably, it has been observed that impairment of cellular functions induced by several anticancer drugs, particularly platinum compounds (182,183), may result in a huge ROS mitochondrial production (270). Nevertheless, although free radicals generated during chemotherapy can potentially target all normal tissues, PNS is mostly subjected to their toxic effects because of both the absence of an efficient

protective barrier and the presence of an high content of mitochondria, largely susceptible to ROS damages and important for the activation of cell death pathways (269).

By the way, previous evidences reported that the impaired repair of single strand breaks, like those induced by ROS, may promote the death of non-proliferating cells towards the block of cellular transcription (271).Moreover, as the increase of both ROS levels and DNA adducts induced by platinum compounds treatment correlates with the accumulation of lesions in sensory neurons (31), it has been recently proposed another mechanism to explain the onset of peripheral neuropathies. Notably, it has been hypothesized that the high amount of ROS platinum-induced could be physically responsible for oxidative DNA damages arising in neuronal cells, leading to the development of neuropathy, included demyelination, microtubular damages and apoptosis (199,272).

To support this hypothesis, some works demonstrated the involvement of BER pathway in mediating neurotoxicity of platinum derivatives, particularly cisplatin: specifically, an efficient repair of oxidative lesions through BER mechanism is able to counteract the onset of peripheral neuropathy (27,187,268). Notably, an important role in this mechanism played by key BER enzymes as Ape1: it has been demonstrated that the PNS sensitivity to cisplatin increases interfering with the DNA repair activity of this protein, that results significantly up-regulated in sensory neuronal cells upon cisplatin treatment probably suggesting a possible protective attempt of the neuronal cell (27,187).

In conclusions, the role of BER pathway in the maintenance of genome integrity and stability is particularly relevant in nervous system, differently exposed to DNA damaging agents potentially causing significant neuronal dysfunctions.

2.4 Targeting BER to enhance cancer therapy

Almost of the chemotherapeutic agents used in cancer therapy aims to affect the cell cycle progression either blocking proteins involved in DNA synthesis or inducing a big amount of DNA damages, thus promoting cell death (5).

These specific strategies exploit an essential feature of cellular physiology: in fact, as for normal cells, also for tumor ones, DNA integrity is an essential step for the following cell replication. Normally, specific checkpoints proteins, like *p53*, ensure the

temporary block of cell-cycle progression thus allowing the efficient repair of possible genome lesions before its replications (273,274). Therefore, although cancer cells usually exhibit a typical predisposition to escape the cell-cycle checkpoints mechanism (5), the recruitment of DNA repair systems is essential to ensure them an efficient proliferative rate (3).

Nevertheless, more evidences support an impairment of at least one of the DNA repair mechanisms in several types of cancer (5,275): despite this feature could potentially sensitize tumors to therapy, indeed other mechanisms promote its survival. By the way, it has been demonstrated that cancer cells use alternative repair pathways to bypass the DNA lesions induced by chemotherapeutic drugs (23,141,142): a classic example is represented by the enhancing of the trans-lesion synthesis mechanism activated against cisplatin adducts (168,163). As a consequence, thanks to these and other escaping systems, tumor cells can easily counteract the cytotoxicity of several chemotherapeutic treatments, developing a resistant phenotype that heavily restricts the therapeutic efficacy of anticancer drugs, preferentially of those targeting DNA (23,142).

Therefore, to overcome cancer cell resistance, it is clearly necessary to develop novel compounds that exploit a different and/or complementary damaging mechanism, potentially promoting a combined therapeutic approach (5). At the same time, knowledge of the molecular mechanisms involving the activation of DNA repair pathways as a consequence of chemotherapeutic agents is of paramount importance for designing more effective cancer therapeutic strategies.

To these aims, the interest in targeting DNA repair pathways for cancer therapy is recently emerging (2,3).

Notably, several evidences indicate BER mechanism as a promising candidate to get this purpose: beside its association with cancer resistance phenomena (22,29,276), BER pathway is the pre-replicative DNA repair mechanism, usually activated for the removal of both base lesions and single strand breaks (3). In addition, since BER mechanism plays a critical role in the maintenance of genome stability and integrity thus ensuring cell cycle progression as well as cell survival (210,211,222,247,248), it could be hypothesized that its role in DNA lesions processing is essential also to promote cancer cell progression (3). This means that targeting BER mechanism efficiency could potentially enhance the cytotoxic effect of drugs damaging DNA. Furthermore, the recent evidences for a possible intrinsic coordination of BER proteins (277–279) exhorts to identify a specific BER protein that could be

preferentially targeted to significantly affect cell survival, towards impairment of the whole pre-replicative DNA repair BER-mediated.

Although DNA polymerases BER involved, particularly Polβ, could potentially appear as the ideal target to achieve this purpose, some evidences demonstrated that an impairment of its expression and/or activity provokes controversial effects on chemotherapy efficiency (177,177,179,180,280,281). In addition human cells own at least 11 different cellular DNA polymerases performing trans-lesions synthesis (282,283) which overexpression has been already associated with cancer resistance (284,285): thus the inhibition of BER polymerases could be potentially bypassed thanks to the activity of these specific type of polymerases that may accomplish DNA replication, nullifying the aim of BER polymerase targeting.

Moreover also the impairment of DNA ligases could be heavily limited. Notably, the key role of LigIII in repair of both mitochondrial and nuclear DNA lesions suggests that its specific inhibition could probably result toxic also for normal cells (286), despite the recruitment of LigI may partially cope its inactivation (287). Nevertheless, although this evidence indicates that the block of LigI activity could mostly affect BER mechanism, its involvement in DNA replication processes (219) restricts the possibility to inhibit this protein because of the potentially toxicity of its impairment on normal dividing cells (3).

Because of the high limitations observed in targeting the last steps of BER mechanism, the possibility to inhibit the pathway in the first phases, impairing either the glycosylase or the endonuclease activity of the involved enzymes has been recently analyzed.

In particular, the knockout and/or the inactivation of DNA glycosylases are not significantly related to the appearance of a toxic effect as well as to the onset of cancer phenotype in normal cells (288). However, it has been suggested that the inhibition of some of these enzymes, which overexpression is strictly related to survival of specific type of tumors, could potentially lead to an increased sensitivity to cancer therapy (289).

This possibility could be further exploited in the case Ape1, which is emerging as a new candidate target for cancer therapy. Notably, for this protein a direct association between its overexpression and the presence of a cancer resistant phenotype in several type of tumors has been demonstrated (22,27,290–292). These evidences suggest that designing of strategies targeting Ape1 could have a large field of application, potentially solving problems of therapy efficacy toward reduction of

resistance phenomena in various tumors. Nevertheless, although the evidence for embryonic lethality following its knockout (210) could probably restrict the type of methodologies used to target Ape1, its multifunctional nature could potentially represent a solution to this problem. Therefore, it could be highly interesting to evaluate the effect of a specific impairment of Ape1 functions in tumor cells in order to sensitize to cancer therapy.

2.4.1 Ape1 as potential target

Beside its association with phenomena of cancer cell resistance (27,29,290,291,293,294), the involvement of Ape1 in different physiological cellular processes as well as in the development of several pathologies, included neurodegenerative diseases, supports the rational to target this BER enzyme.

Ape1 functions

Although the small size of this protein, composed of only 318 amino acids, Ape1 is essential for mammalian cell viability: while the redox function contributes to modulate cellular responses in oxidative stress conditions (295), the DNA repair activity ensures the maintenance of the genome integrity (24). In addition to these canonical functions, other non-canonical features have been ascribed to this atypical DNA repair protein (Fig. 8). It acts as transcriptional repressor binding specific regulatory sequences, named negative calcium responsive elements (nCaRe) thus regulating the expression of specific genes (296–298). Furthermore, recently, an unexpected role of the protein in RNA metabolism has been observed, as demonstrate by its involvement both in the processing of the abasic rRNA and in the cleavage of c-myc mRNA, supporting a potential Ape1 role also in the quality control of RNA molecules (299–301).

Notably, the RNA binding activity is related to the evolutionary acquired unstructured tail formed by the first 33 amino acids of the protein N-terminal domain (302,303). This region contains the nuclear localization signal (NLS) and results mainly involved in protein-protein interaction as well as in the modulation of the AP site enzymatic activity (304,305).

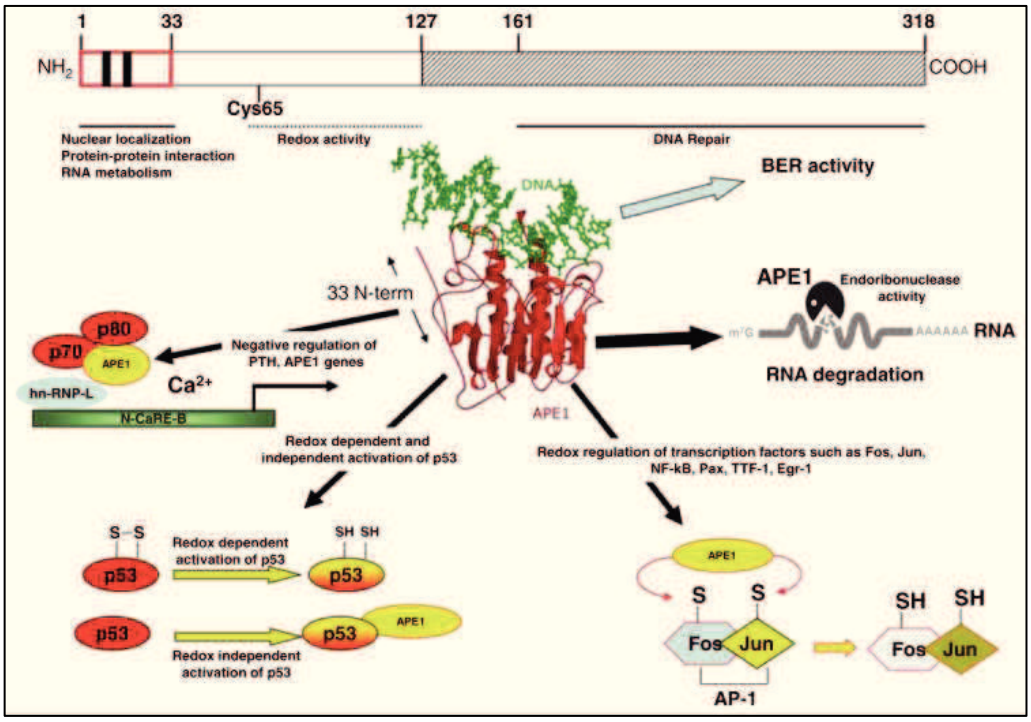

Figure 8: ***Cartoon presenting the different Ape1 functions, canonical and non canonical, and the relative domains*** (Image from Tell et al, *Molecular and Cellular biology* 2010).

Two different acronyms are normally used to refer to this multifunctional protein (Fig. 8), namely Ape1 and/or Ref1, specifically reflecting its dual canonical and essential function in mammalian cells (24). In particular, the key role played in DNA repair is ascribed to its AP endonuclease (Ape1) activity (46), while the acronym Ref1 (Redox Effector Factor-1) is due to its capability of redox activate different transcription factors (TFs), regulating the cellular pattern of gene expression in oxidative stress conditions (306).

These activities are spatially separated in the two functional independent domains of the protein: thus, the highly conserved C-terminal region catalyzes the cleavage of the abasic sites, while the N-terminal promotes the reduction of TFs (279,280,281).

Notably, two specific features contribute to delineate the Ape1 key role in BER pathway: beside of its endonuclease activity over the AP site, that is an essential step of this mechanism crucial to ensure cell survival avoiding persistent unrepaired AP sites (309), Ape1 interacts with downstream BER proteins as Polβ, XRCC1 and FEN-1, thus contributing to coordinate BER reactions (48,310).

In addition to the AP endonuclease activity, the active site pocket of its DNA repair function can be exploited to perform also phosphodiesterase, phosphatase and 3'-5' exonuclease reactions (307,311). Ape1 contains several critical amino acids residues defined through mutagenesis analysis (312,313), that are coordinated by at least one

divalent ion of Mg^{++} acting as an essential cofactor to improve the Ape1 endonuclease activity (25), thus resulting quite strong respect to the 3'-5'exonuclease one (307,311).

Regarding the Ape1 N-terminal domain, it presents specific cysteine residues essential for the redox activation of several TFs. Notably, Ape1 promotes the DNA binding of different proteins as *p53*, AP-1, HIF-1, NF-κB and CREB, maintaining specific cysteine residues within their binding domain in a reduced active state (306). Although the Ape1 N-terminal region presents different thiol groups functional for this reduction (Cys93, Cys99 and Cys138), that of Cys65 is the mainly employed (302). Nevertheless, since Cys65 is located into a hydrophobic pocket resulting unlikely accessible from the TFs (314), it has been suggested that Ape1 may partially modify its conformation in order to expose the thiol group of Cys65 residue and bind the TF, thus inducing its reduction (302).

Almost of the TFs Ape1-activated lead to the expression of genes involved in an adaptive cellular response induced by sub-toxic levels of ROS, specifically promoting various cellular processes as cell survival and growth signaling (28,315). Although these signals can *per sè* promote the development of cancers (13), it has been observed a further role of Ape1 in the progression of tumors, as supported by the activation of YB, a TF inducing the expression of Multi-Drug Resistance gene (MDR1), related to chemotherapy resistance phenomena (316,317). Furthermore, a possible involvement in neuronal cell survival and aging has been ascribed to Ape1, because of its association with the expression of Werner protein as well as Pituitary adenylate cyclase-activating polypeptide (PACAP), respectively related to aging and neuroprotection mechanisms (316–319).

Therefore, although the Ape1 knockdown is lethal (210), the essential role of both these Ape1 functions, namely DNA repair and redox activity, supports the possibility to exploit however this protein as therapeutic target, potentially modulating specific aspects of both carcinogenesis and neurodegenerative diseases.

Notably, given the spatial disposition of its functions, it could be possible to specifically target one of the two active sites interfering with only one activity, without affecting the other Ape1 functions. By the way, different Ape1 inhibitors have been developed (32–34), although several analysis are necessary to evaluate their possible therapeutic feasibility.

Ape1 functional inhibitors

Some evidences suggest that the development of specific strategies targeting Ape1 could potentially enhance the efficacy of conventional cancer therapy, possibly reducing tumor resistance to several drugs treatments (27,29,290,291,293,294). To date, several Ape1 inhibitors targeting mainly either the DNA repair or the redox activity of the protein have been developed (32–34).

Methoxyamine

Methoxyamine (MX) is considered an indirect inhibitor of Ape1 because it not interacts directly with the protein blocking its DNA repair activity but binds the DNA AP site avoiding its processing (32). In particular, MX interacts with the aldehydic C1 atom of the abasic site generated upon removal of the damaged base by a DNA glycosylase, thus creating a stable covalent adduct MX-AP site. As a result, the formation of this intermediate prevents the following binding of BER proteins and the consequent repair of the AP site, particularly avoiding its cleavage by Ape1 (320). Although MX is not significantly cytotoxic *per sè*, its addition to therapeutic agents potentiates the effect of other cytotoxic compounds as the alkylating temozolomide (TMZ) used for the treatments of aggressive gliomas (321). Nevertheless, the use of this compound is strictly limited by both the impossibility to improve its efficacy towards further derivatization of the simple structure (Fig. 9A) and the need to use high concentrations of this drug (20-50mM) to obtain a sensitizing effect in combination with other damaging compounds (28).

Compound #3 and compound #52

Recently, a great efforts has been done to identify specific molecules directly acting on Ape1 endonuclease activity over DNA (33,322). To date, the more promising molecules are the N-(3-(benzo[d]thiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno [2,3-c]pyridin-2-yl)acetamide, named compound #3, and its analog the compound #52 (Fig. 9B, C).

Analysis performed with these drugs show a dose-dependent reduction in the formation of the complex Ape1-DNA, supporting their possible role as competitive inhibitors for the interaction with the DNA binding site of the protein (33). However, the addition of either compound #3 or #52 to other treatments, as TMZ, induces a significant increase in the number of AP sites, thus demonstrating the effective inhibition of Ape1 endonuclease activity. Furthermore, these combined treatments

point out a synergic effect, as demonstrated by the greatly enhanced cell sensitivity to TMZ in combination with both compound #3 and #52 (33).

Moreover, interestingly, several features support the potential therapeutic application of these drugs, as the favorable cell permeability and the good tolerability observed upon application in animals (33), as well as their capability to cross the blood brain barrier (BBB). Notably, this last feature, joined to the reported sensitization to TMZ treatment in combination with these compounds, evidences the potential possibility to use this new approach of combination therapy for the treatment of neuronal cancers, specially the highly aggressive gliomas. Notably, since an overexpression of Ape1, in several adult and pediatric gliomas, with an increase of about 5-10 fold of its DNA repair activity has been reported (294,323), the use of these drugs in combination therapy could potentially enhance the sensitivity to the treatment with the conventional therapy. Particularly, while the hydrophilic nature of compound #52 partially interferes with the BBB penetration, the more lipophilic compound #3 better crosses the BBB, supporting a relevant potential for a specific use of these drugs depending on the type of cancer to treat (33).

E3330

The more used redox inhibitor of Ape1 is E3330 (Fig. 9D). This drug blocks the redox activity of Ape1 regardless of the downstream target, as supported for example by the inhibited activation of HIF-1α, AP-1 and TNF-α (42,324) upon treatment with this compound. Furthermore, E3330 treatments affect both tumor cell proliferation but also capillary formation, efficiently interfering with cancer progression (325).

Analysis of the E3330-mediated mechanism of Ape1 inhibition point out the lack of formation of covalent adducts, that have been instead observed in the presence of E3330 analogues (34). Nevertheless, it has been recently proposed a new mode of interaction between E3330 and Ape1, potentially explaining the inhibition of its redox function.

It has been demonstrated that, physiologically, a folded and a partially or locally unfolded conformation of Ape1 may coexist. Notably, this last exposes the normal buried Cys65 residue, preferentially employed for the interaction with TFs (302,326). Upon addition of E3330, the drug interacts and stabilizes the unfolded conformation of the protein, shifting the conformational equilibrium toward this Ape1 state (326). Although this perturbation of the conformational equilibrium could be similarly induced by the physiological interaction with oxidized TFs, E3330 promotes also the

activation of Ape1 cysteine residues favoring the formation of disulfide bonds, specifically involving Cys65 and or Cys93 (302,327). Since these residues are normally unaccessible in the folded conformation of Ape1, the induction of disulfide bonds formation occurs in the portion of locally unfolded conformation of the protein: this means that the Cys65 thiol group is not available to perform its redox activity, thus effectively decreasing the rate of Ape1 molecules able to reduce TFs (326).

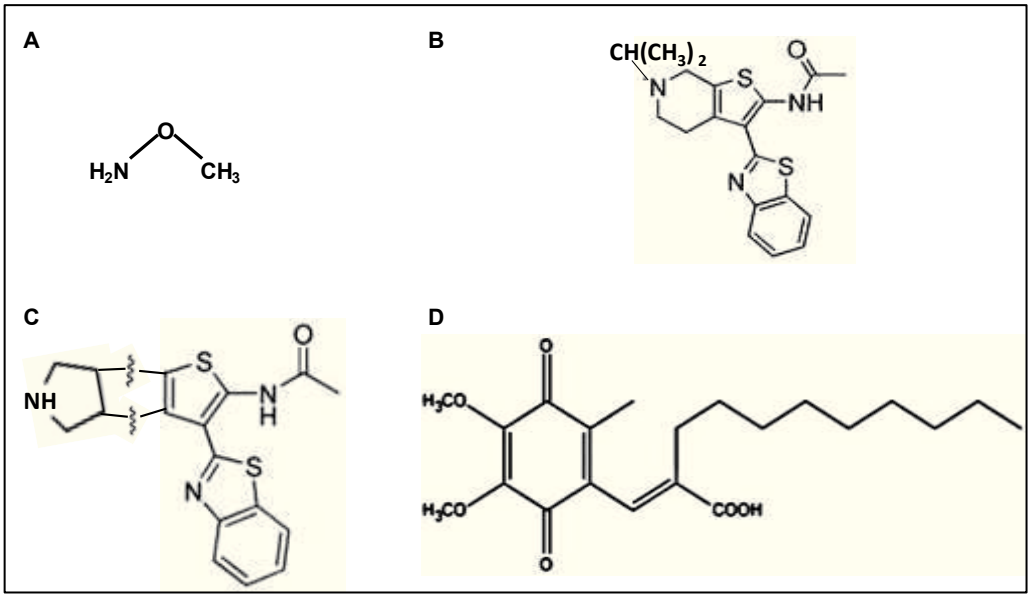

Figure 9: ***Ape1 inhibitors.***

The image reports the structure of the Ape1 inhibitors used in the work: methoxyamine (A), compound #3 (B), compound #52 (C) and E3330 (D) (Image adapted from Rai et al, *Journal of Medical Chemistry* 2012; Kelley et al, *Antioxidant & Redox Signaling* 2011).

3 Results and discussion

3.1 Ape1 gene silencing affects BER proteins expression in neuronal cancer cells

Although the DNA repair pathways play a key role in the maintenance of genome integrity, they also represent one of the main mechanisms responsible for the onset of cellular resistance phenomena to chemotherapeutic treatments (276,328–330). Accordingly, several reports show an increased expression of some of these repair proteins in chemoresistant cancer cells proving also an apparent correlation between an increased cellular sensitization to genotoxic agents and the reduction of their expression levels (29,290,291,331). Therefore, the interest in developing strategies aimed at targeting DNA repair proteins is growing. This aim is essentially pursued through development of direct inhibitors (i.e. small compounds) or gene silencing approaches (i.e. RNA interference), in order to impair a specific repair pathway, thus sensitizing tumor cells to chemotherapy.

To this aim, Ape1 protein seems a good and promising target. In fact, Ape1 expression levels are closely intertwined with the sensitivity of different tumors to chemotherapeutic treatments (22,29,291), including highly resistant cancers, as neuronal tumors.

Nevertheless, in neuronal cells the DNA repair pathways, and in particular BER mechanisms, are essential also to counteract the effects of oxidative stress and environmental pollutants (19,266,299,329,332,333), commonly identified as contributory causes of neurodegenerative disease and aging. This is also demonstrated by the presence of alterations both of the expression levels and of the functions of DNA repair proteins, in particular of BER proteins and Ape1, in different neurodegenerative diseases as well as in aged tissues (257,263,266,299,334).

For these reasons, a good knowledge of the mechanisms regulating the expression levels of DNA repair proteins is necessary to develop a strategy to specifically modulate them and consequently their response to damaging agents, depending on the biological conditions of different tissues.

However, despite the interactions existing among DNA repair proteins, such as BER proteins, suggest the existence and need of fine tuning mechanisms regulating their

functions to better coordinate the damage repair, the mechanisms regulating their expression levels are still poorly understood. In particular, although the key role of Ape1 in neuronal tissue, both to reduce the oxidative stress damages and to sensitize to chemotherapy treatments, is evident, no information are available regarding a possible effect of Ape1 expression on the other BER enzymes. This hypothesis is of particular relevance in light of the role of Ape1 as a transcriptional coactivator (306), as well.

Starting from the sensitizing effect to chemotherapeutic treatments, reported in different cancer cells, upon Ape1 silencing (291,335), it may be hypothesized that the reduced viability could be also related to a possible dysregulation of the overall BER pathway induced by Ape1 knockdown.

To address this issue, the SF-767 glioblastoma cell line was used and Ape1 knockdown was obtained through RNA interference (RNAi) technology by means of small interference RNA (siRNA) transfection. A time course analysis, starting from 24 hours upon silencing, was performed to evaluate the expression levels of BER proteins through Western blot assay (Fig. 10A). Ape1 depletion resulted in a general down-regulation of all BER enzymes expression upon 24 hours of silencing, while the protein levels appeared differently regulated after 36, 48 and 60 hours. Remarkably, the stronger effect was observed on polymerase δ, that remained constantly reduced, at each time point, compared to the control cells (SCR) (Fig. 10B).To confirm the Ape1-dependent dysregulation of BER proteins, the same experiment was performed on HeLa cells. Also in this case, a reduction of all BER proteins was reported after 24 hours of Ape1 silencing (Fig. 10C), again with a more pronounced effect on polymerase δ expression (Fig. 10D).

These results supported the hypothesis that a dysregulation of the whole BER pathway (BERosome) stability could be induced by the down-regulation of Ape1 and that it can be observed also in other cancer cell types, suggesting a cell type-independent effect of Ape1 levels on BER proteins expression. Moreover, data not shown clearly demonstrated that these effects were not due to the specific siRNA sequences used for the experiments, since similar data were obtained through different RNAi strategy based on inducible expression of Ape1-specific short harpin RNA (sh RNA) (personal communication by Dr. Lisa Lirussi).

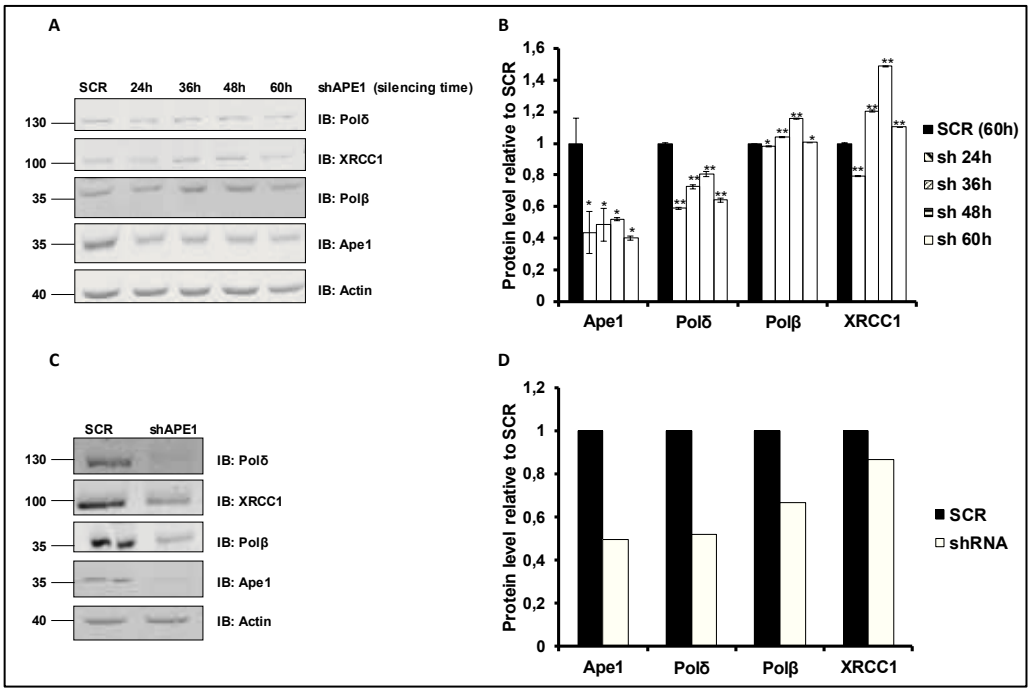

Figure 10: ***Ape1 silencing induces a reduction of BER enzymes expression level*****.**
SF-767 were efficiently transfected with siRNA to silence the Ape1 gene. Cells were collected 24, 36, 48 or 60 hours post transfection and processed to obtain total cell extract, as reported in Materials & Methods. A) Protein lysates were analyzed through Western blot to detect the expression levels of Ape1, Polymerase δ, Polymerase β and XRCC1 protein in SF-767 cells at the different silencing time points. Actin protein level was used as housekeeping protein to normalize samples. B) The histograms represent the mean of three independent experiments of silencing performed in SF-767 cells. Student's t test analysis was used to determine statistical significance. Both ρ-value < 0.05 (*) and ρ-value < 0.001 (**) were considered as statistically significant. C) Representative Western blotting of BER proteins upon 24 hours of Ape1 silencing of HeLa cells. D) The histogram reports the difference between BER proteins levels detected in HeLa cells transfected with control siRNA (defined SCR) and those silenced through transfection with siRNA against Ape1.

In order to define the mechanism inducing the down-regulation of BER proteins, Q-PCR analysis was performed, at the same time of silencing, in SF-767 cells. In this case, mRNA expression levels of the different enzymes was not directly correlated with Ape1 down-regulation (Fig 11). While, as expected, the transcriptional levels of Ape1 were decreased as consequence of the RNA interference activity at each time point, the mRNA levels of BER proteins enzymes were significantly reduced only upon 36 and 48 hours of silencing, showing a different trend at 24 and 60 hours depending on the specific enzyme analyzed. Moreover, the BER proteins expression levels previously analyzed (Fig. 10B) poorly matched with the mRNA levels observed here (Fig. 11): while the expression of both polymerase β and XRCC1 increased, starting from 36 hours of Ape1 silencing, their mRNA levels resulted significantly

reduced; at the same time, the transcriptional levels of polymerase δ were not constantly reduced, as expected by steady down-regulation of the relative protein.

This weird correlation between protein and mRNA levels of BER enzymes suggested that other mechanisms, such as an effect on protein stability, could be involved in the Ape1-related BERosome dysregulation.

Nevertheless, taken together, these data support a possible regulatory effect of Ape1 expression levels on the BER proteins, that could potentially explain the sensitization to chemotherapeutic treatments observed in other cancer cell lines upon Ape1 knockdown.

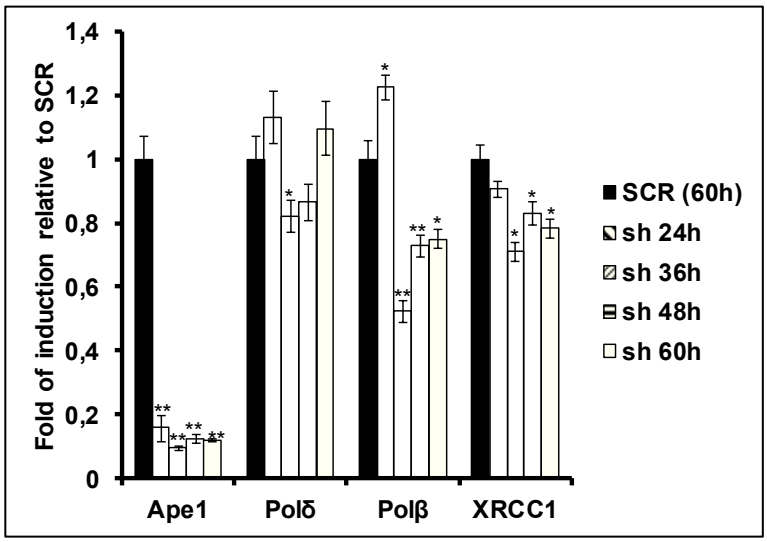

Figure 11: ***Effect of Ape1 silencing on BER proteins transcriptional levels.***

Q-PCR analysis of BER enzymes mRNA levels was performed in SF-767 cells upon APE1 silencing for 24, 36, 48 and 60 hours, performed as above (Fig. 1). Data shown in the graph were normalized to the amount of GAPDH and represented the trend of three independent experiments. Student's t test analysis was used to define significance. Both ρ-value < 0.05 (*) and ρ-value < 0.001 (**) were considered as statistically significant.

3.2 Dysregulation of BER proteins expression is related to the Ape1 DNA repair activity and not to its redox function as a transcriptional co-activator

Given the multifunctional nature of Ape1 and since its several activities, as DNA repair enzyme and redox transcriptional co-activator, are both implicated in the maintenance of cell viability and genome integrity (211), the RNA interference

approach did not allow us to specifically define which of these two Ape1 functions affected BERosome expression.

In order to dissect the involvement of the redox and/or the DNA repair activities of Ape1 in the dysregulation of BER proteins, specific Ape1 inhibitors were used to individually block each function and to analyze the resulting effects on BER proteins expression.

To this purpose, four different drugs were tested: E3330, able to inhibit the redox activity of Ape1 (34) and three different inhibitors (i.e. methoxyamine, compound #3 and #52) specifically targeting its endonuclease activity (32,33). In particular, the latter type of compounds differently prevent the completion of the repair process: compound #3 and #52 directly act on the DNA repair activity of Ape1 by interfering with its binding to the abasic-site (33), while methoxyamine (MX) modifies and stabilizes the AP site in order to block the cleavage of the sugar-phosphate backbone exerted by Ape1 (32).

First, dose-response experiments, with each inhibitor, were performed at 24 hours to define the dose to be used for the further treatments (Fig. 12A-D). The data obtained showed that E3330 interfered with cell viability starting from the dose of 50 μM (Fig. 12A). Among the DNA repair inhibitors, MX resulted the less toxic, as it was able to reduce cell viability of about 50% only, when used at concentrations between 10 and 25 mM (Fig. 12B).

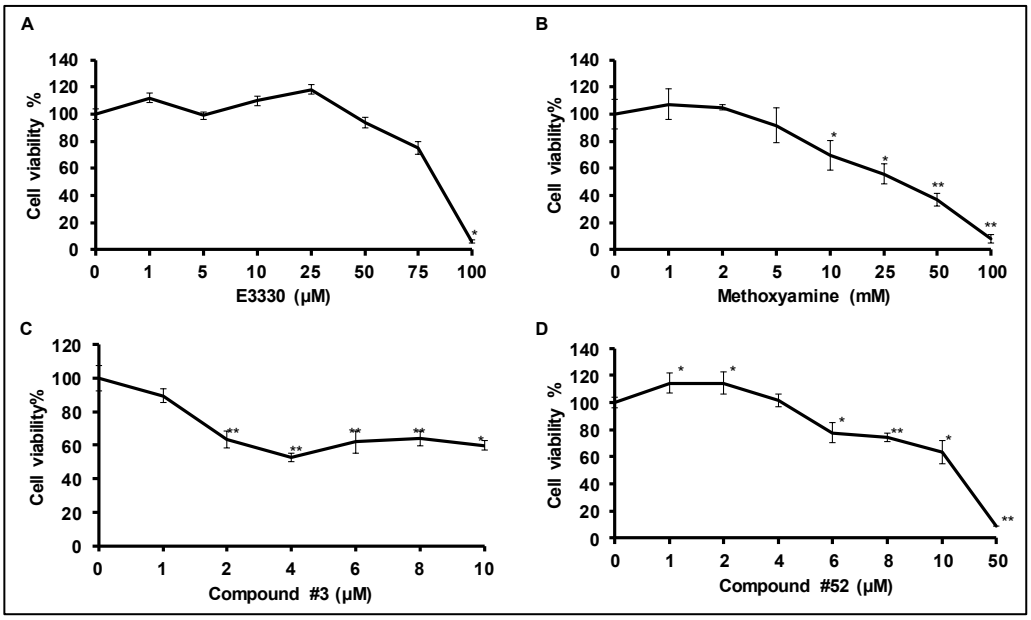

Figure 12: ***Evaluation of Ape1 inhibitors effect on cell viability*****.**

MTS analysis was performed on SF-767 cells upon 24 hours of treatment with the doses showed in the graphs of the following Ape1 inhibitors: E3330 for the redox function (A), while methoxyamine (B), compound #3 (C) and compound #52 (D) for the DNA-repair activity.

On the other hand, the drugs directly interacting with Ape1, i.e. compounds #3 and #52, had a similar trend of efficacy with a reduction of cell viability up to 50% at the dose of 10 μM (Fig. 12C, 12D). Starting from these observations, two different concentrations of each inhibitor where chosen to treat SF-767 cells, in order to have either a mild interference with cell viability or its decrease up to 50%. Upon 24 hours of treatment, cells were collected and total cell extracts were assayed through Western blot analysis to evaluate the expression levels of the BER proteins (Fig 13A-13H).

Data obtained revealed that treatments either with MX or with E3330 induced mild variations on Ape1 expression (Fig. 13A, 13C) and an increase of BERosome levels, with a general slight up-regulation of all BER proteins expression tested (Fig. 13B, 13D). On the other hand, treatments with specific Ape1 endonuclease inhibitors, i.e. compound #3 or compound #52, differently affected Ape1 expression levels: while compound #3 seemed to reduce Ape1 levels at both working doses (Fig. 13E), a mild effect was observed upon treatment with compound #52 (Fig. 13G).

However, both inhibitors were able to reproduce the effect induced by Ape1 knockdown on BER proteins (Fig. 13F, 13H), despite the effect continued to be remarkably more evident on polymerase δ. Taken together, these data suggested that the effect of Ape1 silencing on BER proteins expression was related to the

absence of the endonuclease activity of the protein and not to the redox one. Moreover, interference with the DNA repair activity of Ape1, rather than the block of the AP site repair, was associated with the observed dysregulation of BERosome, as demonstrated by the inability of MX to exert any major effect on BER enzyme expression.

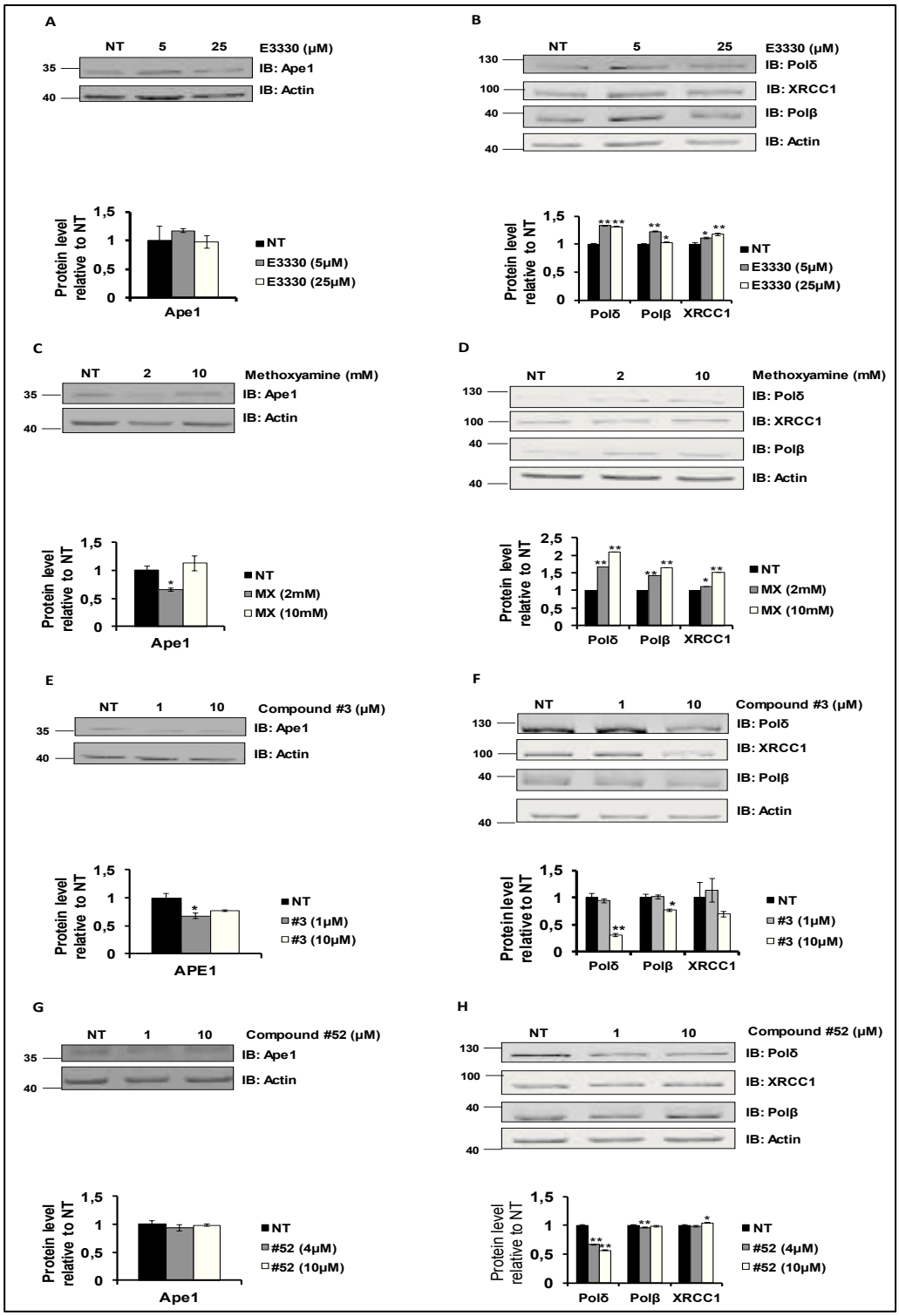

Figure 13: ***Inhibition of specific APE1 functions differently affects the expression of BER enzymes.***

SF-767 cells were treated for 24 hours with different Ape1 inhibitors. The doses used for each inhibitor were chosen based on the dose/response curves showed in Fig. 3. Upon treatments, cells were collected and Western blot analysis was performed on total cell extract to detect the level of APE1 (A, C, E, G), Polymerase δ, Polymerase β and XRCC1 (B, D, F, H). Either actin or tubulin were used to normalize samples. Representative Western blotting analysis was shown for each inhibitor and in the histograms below reported the mean of three independent experiments. The statistical significance obtained with Student's t test analysis was represented in figures: ρ-value < 0.05 (*) and ρ-value < 0.001 (**).

3.3 Inhibition of Ape1 functions differently affects cellular viability upon genotoxic treatments

Given the different effects of Ape1 inhibitors on the overall BER pathway, the use of these drugs could differently affect cellular responses to damages removed by BER mechanisms. In order to define whether and how the interferences with Ape1 activities may modulate the specific response of neuronal cells to different genotoxicants, cell viability was evaluated upon treatment with Ape1 inhibitors in combination with different damaging agents. To this aim, cisplatin, a crosslinking agent, and cadmium, an heavy metal classified as environmental pollutant, were used to treat SF-767 cells. Both agents differently induce neuronal alterations and indirectly promote ROS generation and damages potentially repaired through BER pathway. In particular, while BER activation is involved in the amplification of cisplatin cytotoxicity by mainly affecting the repair of interstrand adducts (173,177,200), cadmium may interfere with the activity of different BER proteins leading to a potential dysregulation of the entire mechanism (26,126–128). Moreover, despite ROS are important for the toxic effect of cisplatin and cadmium, both genotoxicants may cause oxidative DNA lesions, as a consequence of secondary mechanisms rather than to their direct oxidative effect on genome. In fact, the formation of AP sites upon cisplatin treatment seems to be induced by a preferential oxidative deamination of cytosines flanking the guanine interstrand adducts (173), while cadmium leads to an impairment of antioxidant defenses and electron transport chain proteins with consequent “over-load” of ROS and increase possibility of DNA oxidation (97,118).

For these reasons, cellular responses to these genotoxicants were compared with those observed using hydrogen peroxide (H_2O_2), that is a classic stimulus used to activate BER pathway since, being also an intracellular ROS, is able *per se* to oxidize DNA.

SF-767 cells were treated for 24 hours with increasing doses of the genotoxic agents with or without the addition of a fixed dose of each of the Ape1 inhibitors, used at the concentrations previously defined (Fig. 12A-12D). MTS assay was used to analyze the cell viability upon treatment both with the genotoxic agents alone (Fig. 14) or in combination with the different inhibitors (Fig. 15-18).

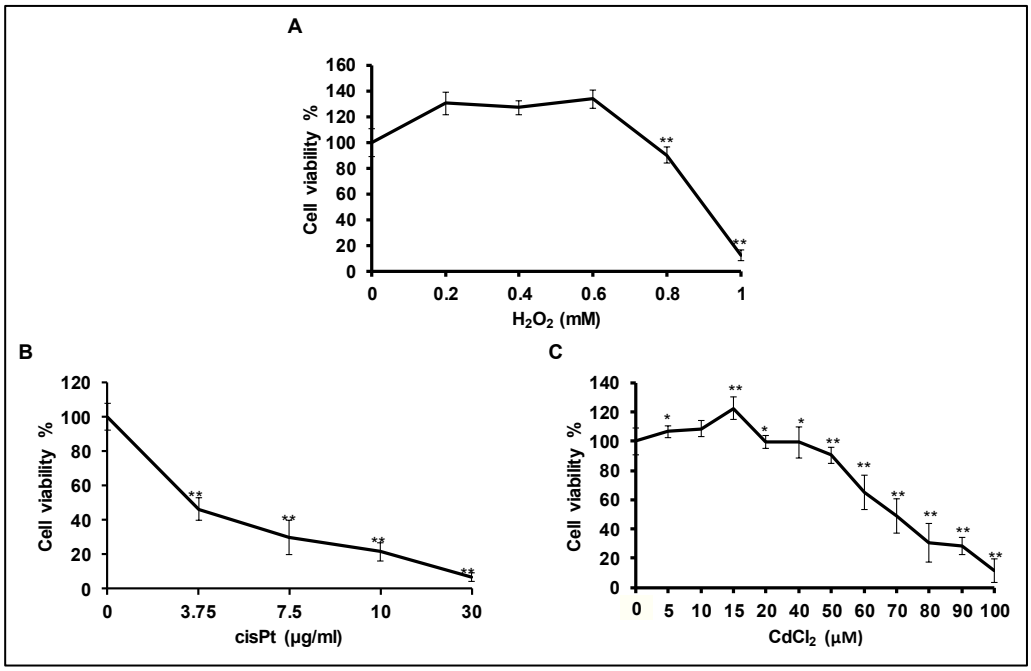

Figure 14: ***Effect of genotoxic damaging agents on SF-767 viability.***

SF-767 were treated for 24 hours with hydrogen peroxide (H_2O_2) (A), cisplatin (B) or cadmium (C) at the doses indicated in figure. Cells viability was tested through MTS assay. The graph is the mean ± SD of at least three independent experiments. The statistical significance obtained with Student's t test analysis was represented in figures: *ρ*-value < 0.05 (*) and *ρ*-value < 0.001 (**).

Dose-response curves showed a basal different sensitivity to each damaging agent used.

While H_2O_2 treatment showed a toxicity starting from the dose of 0.8 mM (Fig. 14A), cisplatin and cadmium strongly affected cell viability (Fig. 14B, 14C) leading to a drastic reduction at lower concentrations. This could be related to the strong cytotoxic activity of both compounds, which is related to a significant amount of alterations occurring at multiple levels and possibly causing a faster kinetics activation of the cellular death pathways. Moreover, while H_2O_2 and cadmium treatments revealed a mitogenic effect at lower concentrations, the doses used for cisplatin treatment immediately induced a drop in cell viability. The biphasic trend, detected upon H_2O_2 and cadmium treatments, may be explained with the activation of proliferative signaling that could potentially explain also the role of cadmium in the development of cancer (94,95).

Therefore, given the different effects of H_2O_2, cisplatin and cadmium on cell viability, it was not surprising to obtain distinct specific cellular responses upon addition of the different Ape1 inhibitors, depending both on the damaging agent and on which Ape1 inhibitor was used. Upon 24 hours of H_2O_2-, cisplatin- and cadmium-treatments with or without Ape1 inhibitors, SF-767 viability was measured and the mean of three

different experiments was analyzed, comparing the effects induced by the damaging agent alone or in combination with the different Ape1 inhibitors (Fig. 15-18).

Differently from what observed with H_2O_2, in which the block of the Ape1 redox function with E3330 severely affected the cell viability (Fig. 15A), the addition of this inhibitor both to cisplatin and to cadmium treatment showed an unexpected mild protective effect (Fig. 15B, 15C). A recent study reports a similar response in sensory neurons, where E3330 reduced the toxic effect of ionizing radiation (268). Moreover, recently, it has been observed that the exposure of sensory neurons to E3330 enhances the Ape1 endonuclease activity (199): this could potentially explain the protective effect of the redox inhibitor that probably, by improving BER activity, may allow an increased repair of DNA damages leading to the potential onset of resistance phenomena. Furthermore, the presence of a protective effect, more evident at low doses of cadmium treatment, may be suggestive of an increased activation of different survival and proliferative pathways (249).

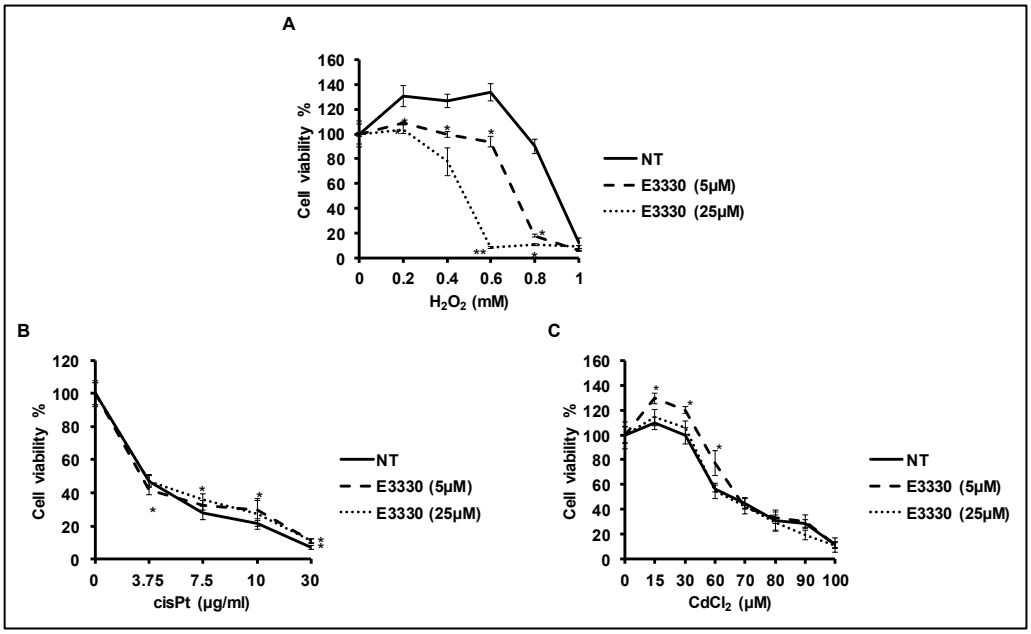

Figure 15: ***Role of the Ape1 redox activity in response to damage agents.***

Cells were treated for 24 hours with genotoxic agents either alone or in combination with two doses of E3330. H_2O_2 (A), cisplatin (B) and cadmium (C) were used at the indicated concentrations. Cell viability was tested with MTS assay. The graphs represent the data from at least three independent experiments. Statistical significance was calculated with Student's t test (*p*-value < 0.05 (*) and *p*-value < 0.001 (**).

Notably, interference with Ape1 DNA repair activity differently affected cellular responses to genotoxic damaging agents, in particular to cisplatin and cadmium treatments. Beside MX, that sensitized cells to all genotoxicants (Fig. 16), both

compound #3 and #52 affected cell viability in an opposite manner, which was strictly related to the combination with cisplatin or cadmium treatments, respectively (Fig. 17, 18).

Anyway, depending on the specific damaging agent used, the sensitization induced by MX reached a different extent (Fig. 16). As expected, both working-doses of MX strongly reduced cell viability sensitizing to H_2O_2 treatment starting from the lower doses of treatment (Fig. 16A). Moreover, despite a mild sensitization effect was obtained with cisplatin co-treatment (Fig. 16B), MX leaded to a severe decrease of cell viability in addition to cadmium treatment (Fig. 16C) with a similar trend to that observed with H_2O_2. Furthermore, the toxicity induced by the Ape1 inhibitor, in the presence of this heavy-metal, revealed a sensitization effect also at lower doses of cadmium, that resulted proliferative *per se* (Fig. 16C).

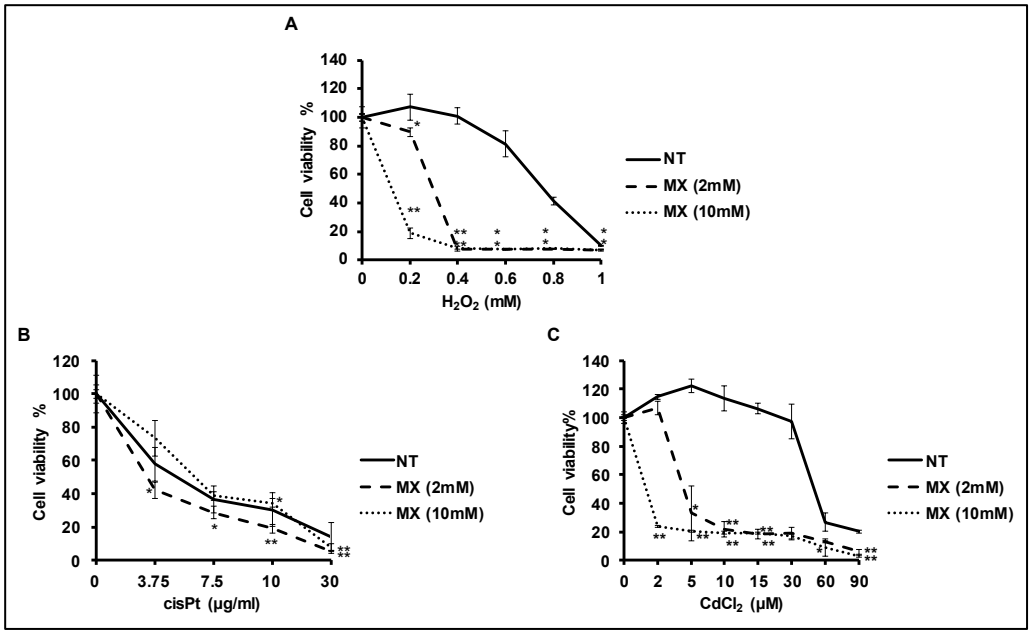

Figure 16: ***Effect of MX on genotoxic treatments.***

SF-767 cells were treated for 24 hours with genotoxic agents either alone or in combination with two doses of MX. H_2O_2 (A), cisplatin (B) and cadmium (C) were used at the indicated concentrations. Cell viability was tested by MTS assay. The graphs represent the viability data of at least three independent experiments. Statistical significance was calculated by Student's t test (*p*-value < 0.05 (*) and *p*-value < 0.001 (**).

This different extent of MX-induced sensitization suggested a distinct involvement of the BER pathway in response to damages induced by cisplatin and cadmium and may possibly be ascribed to the different kind of DNA lesions generated by the cisplatin with respect to those generated by cadmium and H_2O_2 alone. While exposure to cisplatin causes a collateral activation of BER pathways that enhance its

toxicity, mainly caused by DNA intra and interstrand adducts (67,173), cadmium promotes a rapid oxidation of genome quickly altering the intracellular redox homeostasis (336) and thus engaging the BER pathway to fulfill the repair.

Interestingly, as mentioned before, the direct interference with the endonuclease activity of Ape1, both with compounds #3 (Fig. 17) and #52 (Fig. 18), caused an unexpected protective effect once in combination with cisplatin treatment (Fig. 17B, 18B).

Although the cellular viability was drastically reduced, once treating cells with H_2O_2 in the presence of both the inhibitors (Fig. 17A, 18A), a mild reduction was observed in combination with cadmium (Fig. 17C, 18C). Although the effect resulted more apparent by co-treating cells with compound #52 (Fig. 18C), also the #3 exerted a sensitizing effect on cells at higher doses of cadmium (Fig. 17C). Notably, the toxicity induced by blocking the Ape1 endonuclease activity was lower than that observed upon cadmium treatment in the presence of MX (Fig. 16C), probably because of a residual capability of the protein to cleave the AP sites (33), thus avoiding the accumulation of toxic BER intermediates.

A completely opposite effect was observed in the case of cisplatin treatment experiments upon co-treatment with compound #3 and #52 (Fig. 17B, 18B), in which a significant increase of cell viability pointed out a concentration-dependent protection of these drugs toward cisplatin cytotoxic effect. Confirmatory data, concerning this protective effect, were obtained also with SH-SY5Y cells, a human neuroblastoma cell line (data not shown).

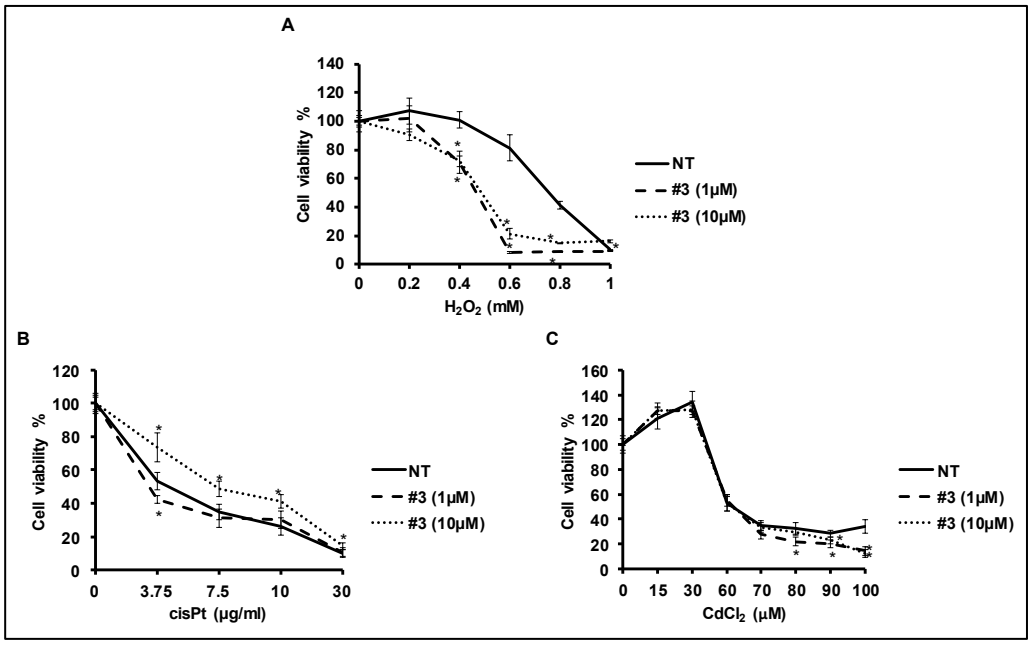

Figure 17: ***Compound #3 differently affects cell viability upon genotoxic treatments.***

Cells were treated for 24 hours with damage agents either alone or in combination with two doses of compound #3. H_2O_2 (A), cisplatin (B) and cadmium (C) were used at the concentrations indicated. Cell viability was tested with MTS assay. The graphs represent the data of at least three independent experiments. Statistical significance was calculated by Student's t test (*p*-value < 0.05 (*) and *p*-value < 0.001 (**).

Although the addition of compound #3 and #52 should be used as therapeutic strategy to sensitize to cisplatin treatment, these data pointed out an opposite effect on neuronal cells. Notably, despite the induction of a protective effect could defend neuronal cells from side effects of chemotherapeutic treatments, such as peripheral neuropathy, the addition of these Ape1 inhibitors to cisplatin therapy could potentially turn into a double edge-sword leading to the activation of resistance phenomena.

In conclusion, despite both cisplatin and cadmium treatments may lead to DNA lesions potentially repaired through the involvement of the BER pathway, taken together these data suggest that cellular responses to these damage agents could be differently affected by inhibition of Ape1 functions. In particular, while both blocking the redox activity, with E3330, and the interference by MX with AP site processing induced similar effects on cell viability, regardless of the damaging agent used for the treatment, inhibition of the Ape1 endonuclease activity with compound #3 and #52 resulted in responses strictly related to cisplatin or cadmium induced damages.

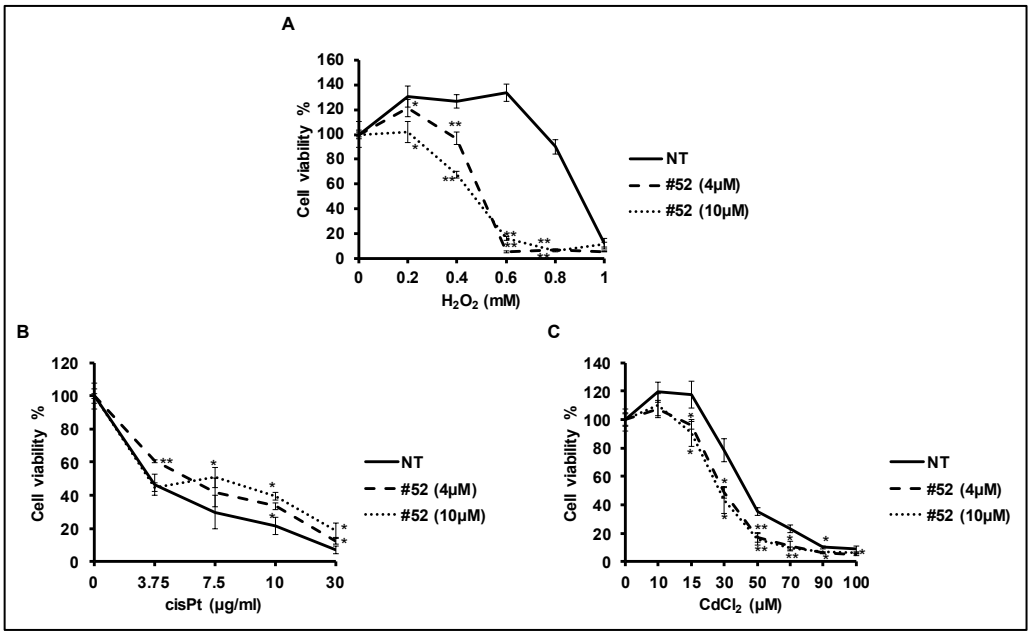

Figure 18 : ***Compound #52 induces similar effects to compound #3 on cell viability upon cisplatin and cadmium treatments.***

SF-767 cells were treated for 24 hours with damage agents either alone or in combination with two doses of compound #52. H_2O_2 (A), cisplatin (B) and cadmium (C) were used at the concentrations indicated. Cell viability was tested with MTS assay. The graphs represent the data of at least three independent experiments. Statistical significance was calculated by Student's t test (*p*-value < 0.05 (*) and *p*-value < 0.001 (**).

Remarkably, these latter evidences have a great relevance in the light of the increasing interest on the opportunity of targeting DNA repair proteins, included Ape1, in order to sensitize cancer cells to therapy. Therefore, although compound #3 and #52 may potentially be used to specifically target Ape1, for development of new anticancer strategies based on their sensitization activity, the induction of completely opposite effects, depending on the damaging genotoxic agent, represents a great limitation. Therefore, further experiments are required to delineate the mechanisms underlying these distinct effects, in order to better define the consequences of using these compounds to block Ape1 endonuclease activity and generalize their use as adjuvant in chemotherapy regimens.

3.4 Genotoxic treatments affect BER proteins expression in SF-767 cells

Considering the data described above, compound #3 and #52 are the only Ape1 inhibitors able to induce both a complete dysregulation of BER proteins expression

levels and an opposing modulation of cell viability in response to cisplatin and cadmium treatments. Starting from these evidences, it could be inferred that the same damaging agents may differently affect the BER enzymes expression, further interfering with the efficiency of BERosome, notably in combination with Ape1 endonuclease inhibitors. Moreover, it is of general interest to understand the molecular explanation for the apparent and paradoxical protective effect of compound #3 and #52 treatments with respect to cisplatin toxicity. In order to test the first hypothesis, SF-767 cells were treated with different doses of the damaging agents, chosen in order to maintain a level of viability comprised between 100% and 40% upon 24 hours of treatment (Fig. 19A, 20A). Then, total cell extracts were analyzed, through Western blot analysis, to evaluate the expression levels of those BER enzymes previously analyzed, i.e. polymerase δ, polymerase β and XRCC1, as from Fig. 13.

In accordance with the overall interpretation, the results obtained pointed out a distinct dysregulation of the BERosome, underlining some differences specifically related to cisplatin (Fig. 19) or cadmium treatments (Fig. 20). Although both agents exerted a suppressive effect on the levels of polymerase δ expression (Fig. 19C, 20C), the expression of the other proteins was differently modulated: while cisplatin mildly affected polymerase β and XRCC1 levels (Fig. 19C), cadmium treatment induced a substantial reduction of both the enzymes (Fig. 20C).

These data suggested that Ape1 endonuclease inhibitors could differently affect cell viability because of the differential effect of cisplatin or cadmium treatments on BER proteins expression. However, although the BER repair mechanism could be mostly compromised by addition of both compound #3 or #52 to the treatments, the difference in cell viability could strictly dependent on the role of these pathways in repairing damages induced by cisplatin or cadmium treatments.

In detail, though cisplatin treatment principally causes the formation of DNA inter- and intra-strand adducts, essentially repaired through NER, recently it has been defined an important role of BER mechanisms in mediating cisplatin cytotoxicity, competing with NER for the repair of interstrand adducts (173,177). Notably, these studies demonstrated that, upon oxidation of the flipped cytosine at the site of damage, the lesion is normally processed by BER pathway and a key role is played by polymerase β, that enhances cisplatin sensitivity through maintenance of the inter-strand adducts (173). These data could potentially explain the protective role of Ape1 endonuclease inhibitors (Fig. 17B; 18B) that, by blocking the BER pathway and in

particular the downstream recruitment of polymerase β, may facilitate the repair of inter-strand adducts through NER allowing cell survival. Furthermore, the involvement of this polymerase in mediating cisplatin cytotoxicity supported the activation of the short-pathway of BER: this would imply that, since polymerase δ is recruited in the long-path of BER, its dysregulation, upon cisplatin treatment (Fig. 19C), could not interfere at all with the involvement of BER in mediating cisplatin toxicity. Anyway, because of the correlation between polymerase δ expression level and cell cycle (337), its dysregulation upon cisplatin treatment could probably reflect a cell cycle impairment, that could further contribute to explain the increased viability upon co-treatment with different Ape1 endonuclease inhibitors (Fig. 17B, 18B).

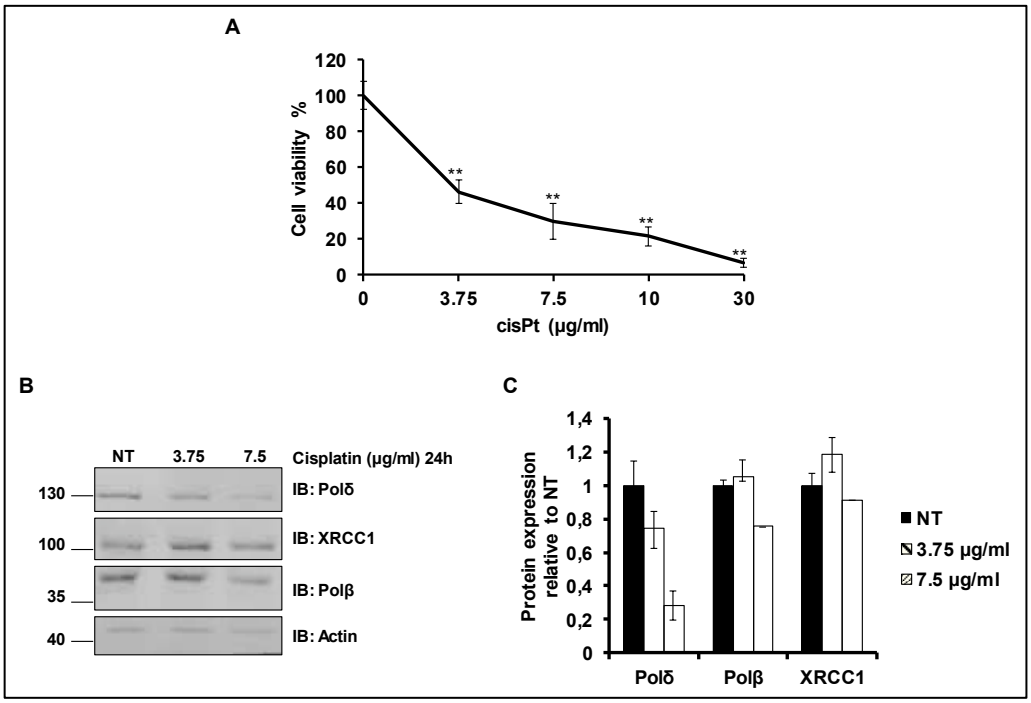

Figure 19: ***Cisplatin treatment reduces the expression levels of polymerase δ but not significantly that of Polβ or XRCC1.***

SF-767 cells were treated with the indicated doses of cisplatin for 24hours. Dose-response curve was obtained using MTS assay to detect SF-767 viability (A). At the same conditions of doses and times, cells were collected and analyzed through Western blot assay for BER protein expression (B). The graph represents the mean of three independent experiments (C). The statistical significance obtained with Student's t test analysis was represented in figures: *ρ*-value < 0.05 (*) and *ρ*-value < 0.001 (**).

On the other hand, the complete dysregulation of BER proteins observed upon cadmium treatment (Fig. 20C) could support the sensitization induced by compound #3 and #52 (Fig. 17C, 18C). Notably, cadmium toxicity is mainly caused by the induction of a strong imbalance of the intracellular redox homeostasis: treatments with this heavy metal have been associated both to an increase of ROS levels and to

an impairment of the antioxidant defenses, leading to an higher oxidation of macromolecules, such as DNA (336). Although the BER pathway could potentially counteract the increased amount of oxidative damages to genome, cadmium may also induce an impairment of the BERosome. In particular, beside the interference with BER proteins activity (26), it has been observed a dysregulation of the 8-oxoguanine DNA glycosylase (OGG1) levels in rat lung cells (128). According to these evidences, also in SF-767, cadmium treatment induced a significant down-regulation of the analyzed BER enzymes (Fig. 20C), suggesting the potential role of this impairment in enhancing cadmium toxicity. To support this, the data obtained in combination treatments with Ape1 endonuclease inhibitors to cadmium treatments showed a further compromise of the cell survival, suggesting a leading role of BER mechanisms in the cellular response to this heavy metal.

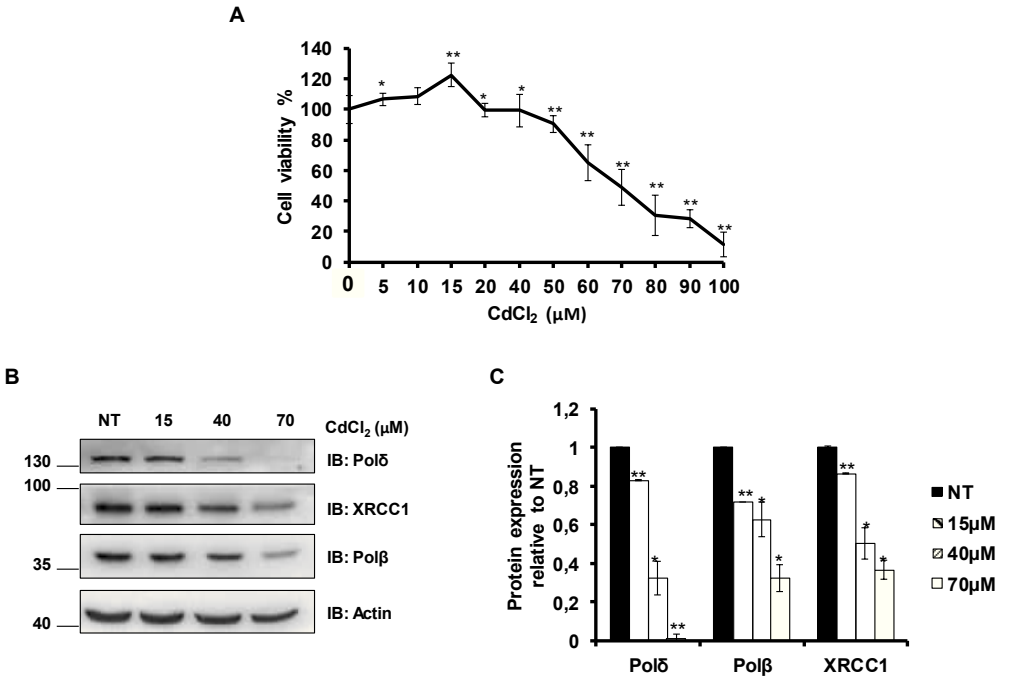

Figure 20: ***Cadmium treatment affects the expression levels of BER proteins, including Polδ, Polβ and XRCC1*****.**

SF-767 cells were treated for 24hours with the indicated doses of cadmium. Dose-response curve was performed using MTS assay in order to evaluate the effect of Cd on SF-767 viability (A). At the same conditions of doses and times, cells were collected and analyzed through Western blot assay for BER protein expression (B). The graph represents the mean of three independent experiments (C). Statistical significance was calculated by Student's t test (p-value < 0.05 (*) and p-value < 0.001 (**).

Taken together, these data demonstrated that the molecular mechanisms underlying the opposite effect of Ape1 endonuclease inhibitors in response to cisplatin or cadmium treatments could be potentially related to the differential effect, in terms of

BER enzymes expression levels, exerted by these genotoxicants, which differently exploiting the recruitment of these proteins. Notably, although BER mechanism is essential to counteract cadmium effects, on the contrary, the activation of this pathway plays a key role in order to enhance cisplatin cytotoxicity. Therefore, also the expression levels of some BER proteins, such as polymerase β and XRCC1, were distinctly affected by these damage agents in order to support their toxic effect.
However, a similar inhibitory effect was observed on the expression of polymerase δ, that was down-regulated by both treatments. Interestingly, the levels of this protein were significantly reduced also by the treatment with Ape1 endonuclease inhibitors compound #3 and #52 (Fig. 13F, 13H). As polymerase δ is also involved in DNA replication processes, its lowered expression correlates with a reduction of cell proliferation and with a lower recruitment in BER pathway: this may potentially represent the main mechanism through which these genotoxicants may exert their anti-proliferative role. Nevertheless, this decrease has been detected also under mitogenic conditions induced by cadmium treatment: using a proliferative dose (15 μM) expression level of polymerase δ resulted significantly down-regulated (Fig. 20C).
In this frame, further experiments are required to better define the mechanisms regulating the opposing effects of compound #3 and #52 on cisplatin or cadmium treatments and to understand how the similar down-regulation of polymerase δ could be related to these distinct phenomena.

3.5 Polymerase δ down-regulation is associated with Ape1 functional inhibition and with genotoxic treatments

In accordance with the hypothesis for a differential involvement of BER pathway in response to cisplatin and cadmium treatments, it was not surprising to observe a distinct effect of these damage agents on BER enzymes expression levels. Although the evidence of a similar effect induced by both treatments on polymerase δ expression could seem paradoxical with the overall idea, the involvement of this enzyme in two distinct cellular processes could potentially explain the effect of its down-regulation regardless of a distinct contribute of BER in response to treatments. In particular, polymerase δ is recruited both in DNA repair, taking part to the long-path of the BER pathway, and in cell proliferation, being responsible for DNA

replication. Starting from these considerations, it could be hypothesized that its reduction may differently affect its involvement in one of the two processes. This hypothesis could possibly give a more complete view of the molecular mechanism underlining the distinct effect on cell viability observed upon inhibition of Ape1 endonuclease activity in combination with cisplatin or cadmium treatments.

3.5.1 A possible explanation for the protective role of compound #3 and #52 in response to cisplatin treatment

The BER pathway is associated with an enhanced cisplatin cytotoxicity thanks to the activity of polymerase β (173,177), supporting the involvement of the short-patch to mediate cisplatin sensitivity. In this context, in agreement with the hypothesis, the reduction of polymerase δ levels induced by cisplatin treatment could suggest a main interference with its recruitment in DNA replication, with a possible impairment of cell cycle. Nevertheless, this effect can possibly be more apparent by blocking BER processing with Ape1 endonuclease inhibitors, that allow a more objective evaluation of the cisplatin “basal” effect on cell viability, without enhancing its cytotoxic effect.
In this view, the strong decrease of polymerase δ observed may be part of an overall cell cycle impairment induced by cisplatin treatment, potentially causing the onset of resistance phenomena upon interference with cellular mechanisms mediating its cytotoxicity, such as BER pathway. Notably, previous works from our group point out that cisplatin treatment induces nucleolar stress as underlined by the formation of fibrillarin caps (338) and demonstrated the relationship among resistance to genotoxic treatments, proliferation impairment and Ape1 relocalization (304). This suggests that, according to the overall interpretation, the induction of a cell cycle impairment by cisplatin treatment may also correlate with an alteration of Ape1 subcellular distribution and thus, its functional impairment. To test this hypothesis, SF-767 cells were treated with cisplatin for 6 hours and the localization of Ape1 was detected by immunofluorescence (IF) assay. As hypothesized, staining for Ape1 showed a significant emptying of nucleolus with an accumulation of the protein in the nucleoplasm (Fig. 21).

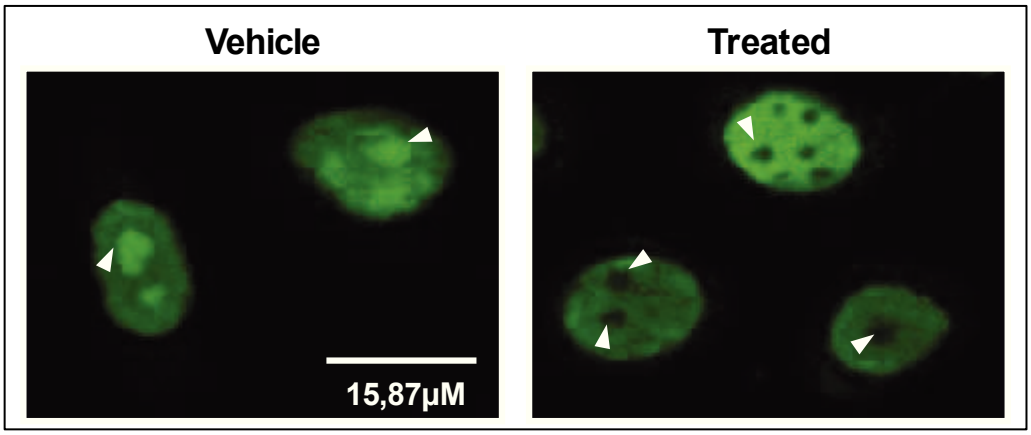

Figure 21: ***Cisplatin treatment induces a relocalization of Ape1.***

SF-767 cells were treated with 30µg/ml of cisplatin for 6 hours. Immunofluorescence assay was performed to detect the localization of Ape1 upon treatment.

Nevertheless, although the observed relocalization of Ape1 from nucleolus to nucleoplasm is physiologically associated to the repair of DNA damages (339), recent evidences (340) and data not shown from our laboratory clearly demonstrate its link with the occurrence of cell cycle impairment. Notably, Ape1 protein is associated with ribogenesis processes in the nucleolar compartment and its relocalization from the nucleolus causes ribogenesis stress (341,342). Notably, an hallmark of ribogenesis stress is represented by *p53* stabilization (342,343). To test this hypothesis, expression levels of *p53* were analyzed as a signal of cell cycle arrest and ribogenesis stress marker. SF-767 cells were treated for 24 hours with cisplatin and then Western blot analysis was performed to detect the level of this marker that, in accordance with the hypothesis, resulted increased (Fig. 22).

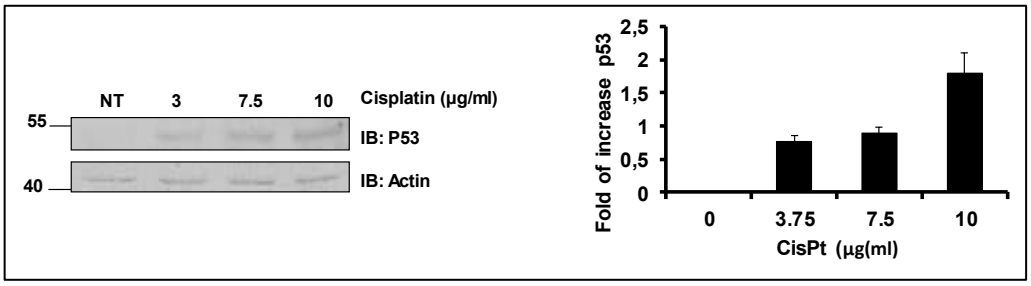

Figure 22: ***Cisplatin treatments up-regulates p53.***

SF-767 cells were treated for 24 hours with the indicated doses of cisplatin. Upon treatments total cell extract were analyzed through western blot to detect *p53* expression level.

Taken together these results support the notion that the reduction of polymerase δ expression levels may be part of a cell cycle impairment induced by cisplatin treatment.

3.5.2 Effect of proliferative doses of cadmium treatment on BER protein expression

Alteration of redox homeostasis (118,336) caused by cadmium is the main responsible of its toxicity. It is enhanced by the induction of a general dysregulation of DNA repair mechanisms, notably BER pathway, leading to a significant impairment of cell viability. In agreement with these considerations, the down-regulation of polymerase δ levels falls in a complete BERosome alteration induced by cadmium treatment (see above). Notably, the result obtained above points out an interesting reduction of all BER proteins also using proliferative doses of cadmium (15 μM; Fig. 20C). Although this result could potentially support the high toxicity of cadmium, the down-regulation of polymerase δ could appear in contrast with the induction of a proliferative effect because of its involvement in DNA replication. On the other hand, the mitogenic effects of low doses of cadmium treatment may be related both to a mutagenic condition and also to an altered expression of protoncogenes (97). Accordingly, the reduction of polymerase δ and the other BER enzymes, observed at proliferative doses of cadmium treatment (Fig. 20C), may result in a lower efficacy of BER pathway, avoiding the removal of oxidative damages and allowing the fixation of DNA mutations, thus affecting genomic stability associated to cell proliferation. Nevertheless, further interference with BER pathway, e. g. by using Ape1 inhibitors, significantly reduces cell viability affecting also the proliferative effect of cadmium. Notably, as compound #3 and #52 application affected *per sé* the overall expression of BER proteins (Fig. 13F, 13H), it could be hypothesized that by adding these drugs to cadmium treatment the efficacy of BERosome may be further compromised. As a consequence, an increased accumulation of toxic intermediates could lead to an higher genome instability causing a reduction of cell survival also at proliferative doses of cadmium.

To test this possible hypothesis, the effective induction of a BER proteins dysregulation also at proliferative dose of cadmium (15 μM) was further evaluated.

Thus, first of all, two different approaches were used to confirm the increase of cell viability with low doses of treatment. Both MTS assay (Fig. 23A) and cell counting assay (Fig. 23B) were performed upon 24 of cadmium treatment, confirming a significant proliferative effect of cadmium at a concentration of 15 μM.

Then, the effect on BER proteins expression levels was defined by treating SF-767 cells with three proliferative doses of cadmium for 24 hours: again the results showed a reduction of the expression levels of all BER enzymes (Fig. 23C, 23D), particularly

of polymerase δ. This result confirmed that cadmium affects the expression level of BERosome also at the proliferative doses. To delineate the extent of this effect, a kinetics experiment was performed upon 2, 6 or 24 hours of cadmium treatment, using the dose of 15 μM. Western blot analysis showed that the pathway was quickly dysregulated, as supported by the down-regulation of all BER proteins levels at 2 hours of treatment (Fig. 23E, 23F). Anyway the strongest effect was obtained on polymerase δ levels that remained steadily down-regulated at each time point.

All together these results confirmed that BER pathway was strongly and quickly impaired by cadmium treatment also at proliferative doses. Furthermore, polymerase δ resulted the BER protein mainly affected by cadmium treatment: notably this fast down-regulation could impair both of its functions, affecting also its recruitment in DNA replication mechanism. Thus, the proliferative effect observed at lower proliferative doses of cadmium may be potentially due to the activation other mechanisms, such as the induction of protoncogenes expression. Moreover, although a reduced amount of polymerase δ may reflect an impaired activation of the BER long-pathway, the later and milder down-regulation of polymerase β could support the removal of part of the oxidative DNA damages induced by cadmium towards the short-pathway of BER. This could contribute to the maintenance of a controlled amount of lesions, favoring the activation of proliferative pathway rather than death mechanisms.

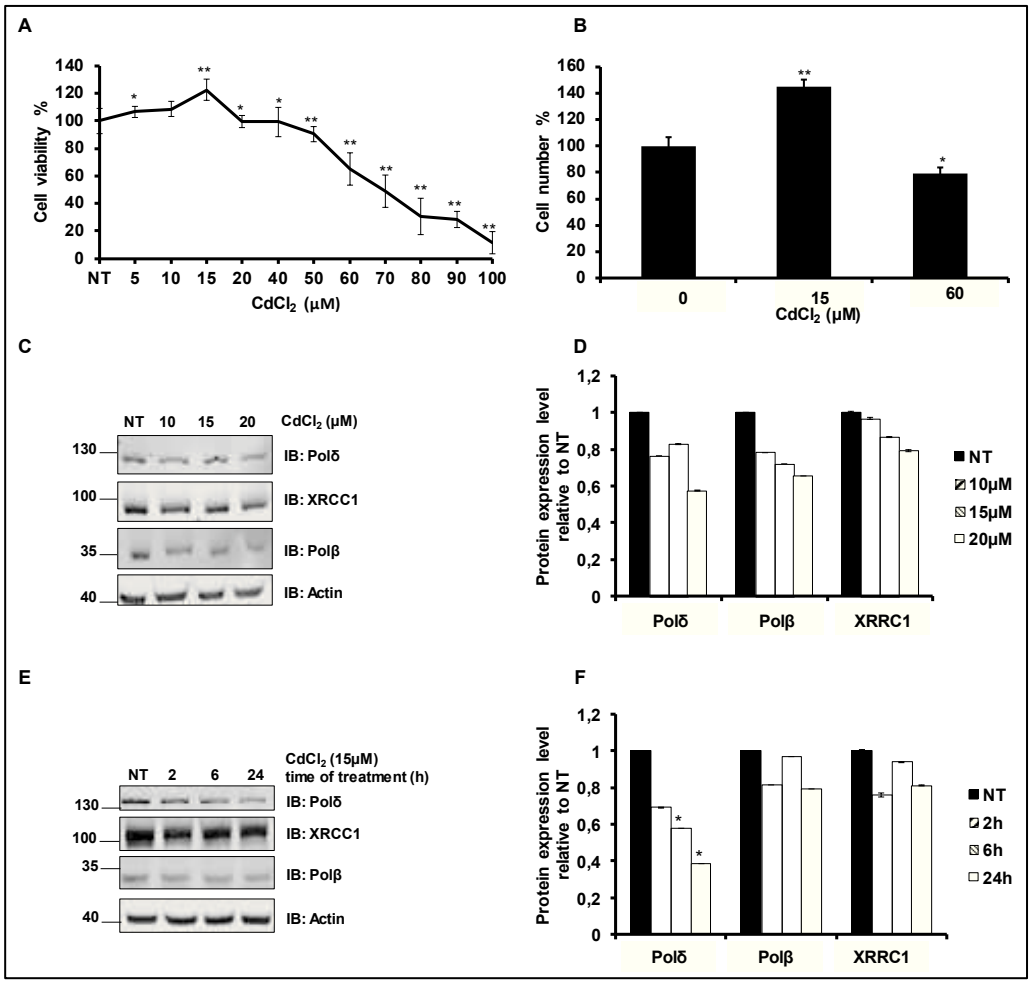

Figure 23: ***Cadmium effect on BER pathway.***

SF-767 were treated with cadmium for 24 hours and cell viability was evaluated through MTS assay (A) and cell counting analysis (B). The effect of low doses of cadmium on BER protein expression levels was evaluated with western blot analysis on total cell extract (C): the relative quantifications were reported as mean of three independent experiments in the graph beside (D). A kinetics analysis of cadmium induced BER enzymes down-regulation was performed at the indicated point time (E) and the mean of three independent experiments was showed in the graph (F). Statistical significance was calculated with Student's t test *p*-value < 0.05 (*).

Taken all together, these data supported the initial hypothesis showing that, despite the similar effect on polymerase δ expression levels, cisplatin and cadmium treatment may differently interfere with the recruitment of this protein either in BER pathway or in DNA replication. In agreement with this interpretation, the collected results suggested that, in both cases, the dysregulation of polymerase δ could potentially fall into a more complex mechanism, including cell cycle impairment induced by cisplatin treatment or an overall BERosome dysregulation related to cadmium application. Moreover, although treatments similarly affected polymerase δ levels, both the modulation of other BER proteins and the effect on cell viability after inhibition of Ape1 endonuclease activity were strictly related to the damaging agent

used: both these evidences supported an opposite role of BER pathway in response to cisplatin and cadmium treatment, respectively to increase cytotoxicity and to protect from oxidative stress.

Nevertheless, although these data added important information in order to define the reasons of the opposite effect of Ape1 inhibitors on cisplatin and cadmium treatment, the molecular mechanism leading to the dysregulation of polymerase δ expression levels remained poorly understood.

3.6 Toward a general hypothesis on the molecular mechanisms responsible for Polymerase δ downregulation by distinct drugs

In order to define the molecular mechanism responsible for the reduction of polymerase δ expression level observed upon treatment with the different genotoxicants (i.e. cadmium and cisplatin or Ape1 inhibitors), first of all we considered the hypothesis for a possible transcriptional down-regulation induced by Ape1 inhibitors, cisplatin and cadmium treatments. As a consequence, the promoter region of the gene *POLD1* (Fig. 24) was analyzed to define the presence of specific transcription factor binding sites, potentially involved in the inhibition of gene transcription. Notably, the promoter of *POLD1* contains a binding site for *Sp1* transcription factor that activates the protein transcription, flanked by two binding sites recognized by *p53* that plays a repressive role on the transcription of the gene by a squelching mechanism on *Sp1* (344).

Figure 24: ***Schematic representation of promoter region of POLD1 gene.***
The image report the binding site of *p53* and *Sp1* showing their respective position.

To define whether the down-regulation of polymerase δ was associated to an increased expression of *p53*, Western blot analysis was performed on total cell extract of SF-767 cells treated for 24 hours with the same doses of drugs able to reduce the level of polymerase δ.

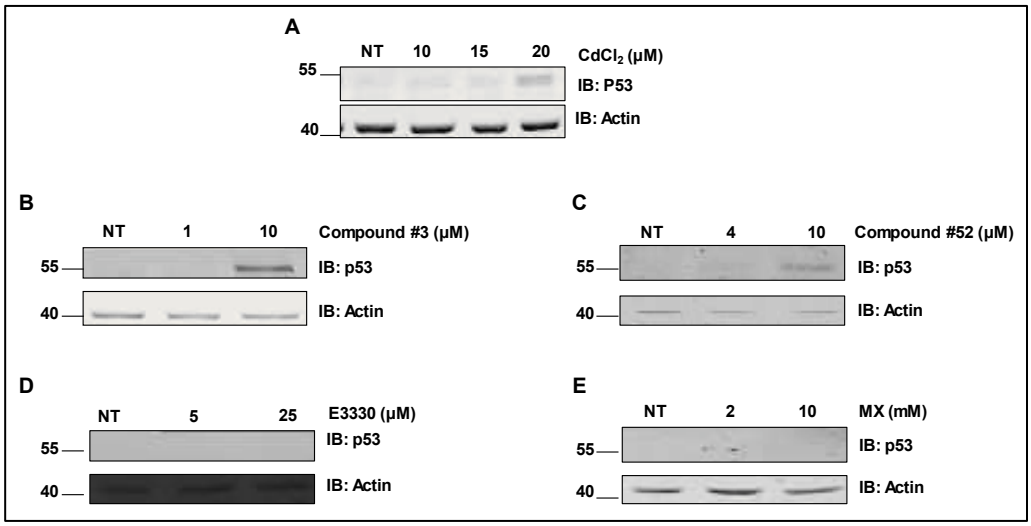

Figure 25 :*Polymerase δ reduction was related to an increase of p53 levels.*

SF-767 were treated with the indicated doses of cadmium (A) or Ape1 inhibitors (B, C, D, E) for 24 hours. Total cell extracts were analyzed through Western blot analysis to detect the level of *p53*. Actin levels were used to normalize samples.

As hypothesized, besides the apparent increase of *p53* upon cisplatin treatment (Fig. 22) both cadmium (Fig. 25A) and Ape1 DNA repair inhibitors (Fig. 25B, 25C) showed an increased level of this protein, which was not observed once using E3330 or MX (Fig. 25D, 25E). To validate the effective presence of a transcriptional repression, Q-PCR was performed on SF-767 cells to evaluate the mRNA levels of polymerase δ upon 24 hours of treatment with cadmium or Ape1 endonuclease inhibitors.

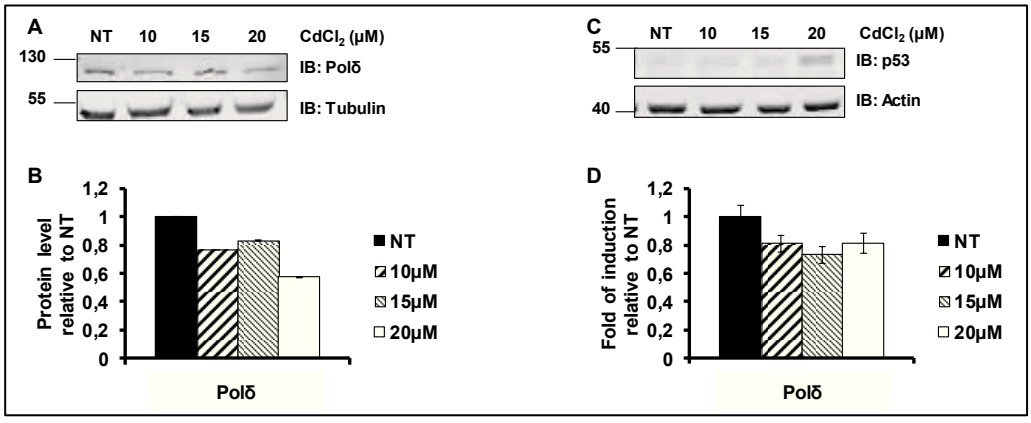

Figure 26: *Cadmium treatment affected the transcriptional levels of polymerase δ.*

SF-767 cells were treated for 24 hours with the indicated doses of cadmium. Cell collected were used to obtain total extract or RNA, respectively analyzed through Western blot analysis to detect both polymerase δ (A, B) and *p53* (C) expression levels and Q-PCR to evaluate the levels of polymerase δ (D). The graphs (B, D) represented the mean of three independent experiments.

As expected, in agreement with the reduction of the protein (Fig. 26A, 26B) and the increase of the transcriptional factor *p53* (Fig. 26C), cadmium treatment induced a similar down-regulation also on mRNA (Fig. 26D), supporting a possible role for this heavy metal in the transcriptional regulation of polymerase δ.

Moreover, when performing the same experiment upon treatment with compound #3 or compound #52, the results obtained were different and in accordance with those obtained in the previous part of this thesis (Fig. 10B-9). Notably, despite these drugs induced both a reduction of polymerase δ (Fig. 27B, 27F) and an increase of *p53* (Fig. 27C, 27G) expression levels, any evident correlation with mRNA levels at the was pointed out in correspondence of the same doses of treatment.

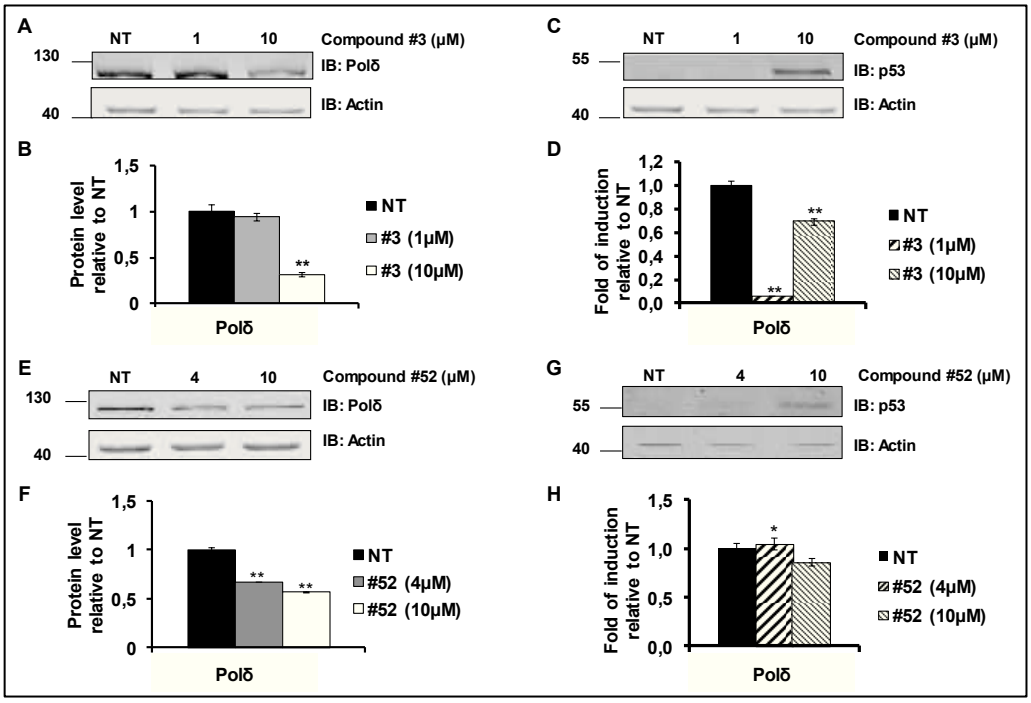

Figure 27: ***Ape1 endonuclease inhibitors differently dysregulated polymerase δ protein and mRNA levels*****.**

SF-767 cells were treated with two doses of Ape1 endonuclease inhibitors, compound #3 (A-D) and #52 (E-H). After 24 hours of treatment, cell were collected used to obtain either total extract or RNA. On total protein extract was performed Western blot analysis to detect both polymerase δ (A, B, E, F) and *p53* (C, G) expression levels, while cDNA obtained from RNA was analyzed through Q-PCR to evaluate the levels of polymerase δ (D, H). The graphs (B, D, F, H) represented the mean of three independent experiments. Statistical significance was calculated with Student's t test *p*-value < 0.05 (*).

Even if unexpected, the lack of any modulation of mRNA levels upon treatment with Ape1 endonuclease inhibitors was in agreement with the effect of Ape1 silencing: notably, although knockdown of Ape1 resulted in a down-regulation of BER proteins,

included polymerase δ, the effect was not mediated by a transcriptional mechanism, as confirmed by the lack of correlation between levels of protein (Fig 10A) and relative mRNA (Fig. 11). Taken together, these data suggested that these Ape1 inhibitors probably induced a dysregulation of polymerase δ expression levels through other mechanisms, potentially involving the stability of the protein.

Nevertheless, to corroborate the inhibitory role of *p53* in the induction of polymerase δ down-regulation upon cadmium treatment, further experiments should be done. Notably, they should be specifically aimed to point out the presence of a possible increased binding of *p53* to the promoter region of the gene *POLD1*, potentially supporting a reduced recruitment of the activator transcriptional *Sp1*.

4 Conclusions & perspectives

Many reports point out the potentiality of targeting DNA repair pathways, specifically BER mechanisms, in order to promote chemotherapy sensitivity with the aim of assisting conventional cancer treatments (2,3,28). In this context, although several evidences support the prospective efficacy to specifically target the key BER enzyme Ape1, data collected here highlight *pros* and *cons* of its functional impairment, at least in neuronal cancer cell lines.

Notably, since the direct inhibition of Ape1 DNA repair activity, through both compound #3 and compound #52 treatments, significantly affects the levels of all BER proteins, the use of these drugs could be potentially exploited to induce a general impairment of this repair pathway thus modulating cancer chemosensitivity. Moreover, this feature could suggest a possible involvement of Ape1, particularly of its DNA repair capacity, in the light of an intrinsic mechanism of BER co-ordination, recently observed in association with the expression levels of other BER enzymes (277–279).

Nevertheless, though this result suggests a possible increase in chemotherapy sensitivity towards specific inhibition of Ape1 DNA repair activity, experiments of cell viability performed in co-treatment with cisplatin disproved this hypothesis, potentially restricting the feasibility of this strategy.

By the way, the protective effect exerted by compounds #3 and #52, with respect to cisplatin treatment, supports the induction of potential resistance phenomena by the use of Ape1 endonuclease inhibitors, possibly related to the role of BER in processing of cisplatin adducts: in fact, the recruitment of BER proteins, upon deamination of cytosines flanking cisplatin lesions, preserves the maintenance of inter-strand crosslink, usually enhancing the cisplatin cytotoxicity (173,177). This would mean that any interference with the activity of BER enzymes could potentially promote the development of a more resistant cellular phenotype, at least toward cisplatin and, possibly, toward crosslinking agents treatment. In addition, it could be hypothesized that the impairment of BER pathway, induced by the direct inhibition of the Ape1 DNA repair activity, significantly reduces the efficacy of this pre-replicative DNA damage repair mechanisms (3), thus inducing a cytostatic effect and preventing

the appearance of the cisplatin cytotoxicity, strictly related to an active cell proliferation (345).

Therefore, although the direct targeting of the Ape1 endonuclease activity could potentially enhance the cancer cell chemosensitivity because of the general impairment of BER pathway, on the other hand the results obtained in combination with cisplatin highlight the need to carefully analyze the effect of combined treatments. Specifically, before using these Ape1 inhibitors in therapy, it could be highly relevant to evaluate the induction of an additional or antagonist effect on cell viability during combined treatments, in order to ensure the expected modulation of cancer cell chemosensitivity.

Nevertheless, the addition of both compound #3 and #52 to cadmium treatment leads to a completely different result, showing an increased sensitivity with respect to the metal alone. This synergistic effect can be ascribed to the essential role of BER mechanism in repairing damages induced by cadmium (101,336); further confirmation was given by the significant reduction of cell viability observed in combination with MX, that stabilizes the AP site avoiding its following processing. Both these results support the key role of oxidative stress in the induction of neuronal cell death mediated by environmental pollutants, as cadmium (30,111), particularly underlining the essential role of Ape1 endonuclease activity in counteracting their cytotoxic effects. Moreover, the significant alteration of BER proteins levels caused by cadmium treatments, in a dose- and time-dependent fashion, supports the presence of an heavy and quick impact on the efficacy of repair mediated by BER, already observed in other reports (26,101,120,126,127).

Interestingly, although the repair of lesions induced by cisplatin and cadmium differently exploit the BER pathway (101,173,177,336), both drugs but also the direct Ape1 DNA repair inhibitors, namely compound #3 and #52, have the same effect on the expression levels of only one of the BER proteins analyzed: polymerase δ. However, since polymerase δ plays a dual biological role, being both recruited in BER pathway (53) and also in DNA replication (337), it could be hypothesized that its down-regulation may differently affect one or both roles of this protein.

Notably, while for cadmium and Ape1 inhibitors treatments the reduction of polymerase δ could be part of a general dysregulation of the BER mechanism, as also suggested by the decrease of the other BER enzymes, probably the down-regulation caused by cisplatin treatment may principally affect the recruitment of this protein on replication processes with consequences on cell proliferation.

Different evidences support that cisplatin treatment could potentially induce a cell cycle dysregulation, as suggested by the detection both of a clear increase of *p53* expression levels and of a strong relocalization of Ape1 from nucleolus to nucleoplasm upon this treatment. Recently, both our results and other evidences demonstrated that cisplatin induces nucleolar stress, potentially causing an impairment of ribogenesis associated to a relocalization of Ape1 to nucleoplasm, thus consequently affecting cell cycle progression (300,303,304,340). Furthermore, these evidences could potentially explain the protective role of Ape1 DNA repair direct inhibitors: in particular, although cisplatin treatment mildly alters the expression levels of the other BER enzymes (polymerase β and XRCC1), the addition of compound #3 or #52 could be sufficient to inhibit the BER-mediated cisplatin cytotoxicity (173,177), thus allowing the appearance of the cell cycle impairment cisplatin induced.

To further corroborate the overall interpretation of a different cytotoxic effect related to the reduction of polymerase δ expression levels, analysis performed upon cadmium treatment show a constant association of its reduction with a general modulation of all BER proteins. Anyway, despite both cadmium and Ape1 endonuclease inhibitors cause a similar alteration of the whole BER pathway with consequences on the repair process, data show that the down-regulation of polymerase δ expression levels is not induced by the same mechanism.

Thus, in order to identify a possible mechanism for cadmium- and Ape1 inhibitors-induced down-regulation of Polδ expression, the promoter region of its gene has been analyzed. Interestingly, it presents two repressor binding sites for *p53* flanking and partially overlapping the sequence recognized by the transcriptional activator *Sp1* (344). Although the increased expression of *p53* detect after either treatments, namely with cadmium and Ape1 endonuclease inhibitors, the mRNA analysis revealed the presence of a decreased expression only upon treatment with the heavy metal, suggesting the possibility for an alternative mechanism of down-regulation of the protein triggered by Ape1 inhibitors, probably involving the stability of Polδ.

Therefore, further analyses are necessary to confirm the presence of a transcriptional mechanism responsible for the down-regulation of Polδ expression mediated by cadmium. Along these lines, the recruitment of *p53* and *Sp1* on its promoter region could be checked through chromatin immunoprecipitation (ChIP) and elettroforetic mobility shift analysis (EMSA), thus evaluating the presence of a competitive binding of the two TFs as already reported for both Polδ and other proteins (344,346,347). As observed in some studies (346,348,349), another possibility is the induction of a

protein-protein mediated squelching mechanism explaining the putative inhibitory function of *p53.* Thus, by exploiting co-immunoprecipitation analysis (CO-IP) the occurrence of this potential interaction between the transcriptional activator *Sp1* and the repressor *p53* could be assayed.

Furthermore, since cadmium has been associated to a carcinogenic role in different cancers (92,97), as supported by the activation of cell growth pathways using low concentrations of this metal (103,106,350), it could be interesting to evaluate the contribution of the Polδ down-regulation, detected at proliferative doses, in the cadmium-related carcinogenesis.

Moreover, the recruitment of low-fidelity polymerases, such as Polη which is activated by *p53* (351), can be also envisioned in order to compensate for the loss of processivity in the replicative mechanism due to the reduction of Polδ expression. This mechanism could represent the basis for an increased mutagenic rate mediated by cadmium. This hypothesis could explain the molecular mechanism triggered by cadmium as a mutagenic compound, supporting its role in cancer and neurodegenerative diseases development. By the way, it has been recently observed that a deficiency of another BER-involved polymerase, i.e. Polβ, is associated to a neurodegeneration phenotype with enhanced Alzheimer's disease features (218) further supporting a leading role of BER in neuronal physiology.

5 Materials & methods

5.1 Cell culture and siRNA knockdown

SF-767 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy), 100 U/ml penicillin, and 10 µg/ml 16 streptomycin sulfate. One day before transfection, cells were seeded onto 6-well plates and transfected 24 hours later with 100 pmol of either a scrambled control siRNA (SCR) or the target-specific siRNA by using the DharmaFECT I Transfection Reagent (GE Healthcare, Milan, Italy) as per manufacturer's indications. The oligonucleotide sequence used for Ape1 was 5'-UACUCCAGUCGUACCAGACCU-3' (Thermo Scientific). Cells were harvested at the indicated time points after transfection.

5.2 Ape1 inhibitors and genotoxicants

The redox inhibitor E3330 (Sigma) was resuspended in dimethyl sulfoxide (DMSO) before use, while methoxyamine (Sigma) was dissolved in PBS. The other nuclease inhibitors, compound #3 and #52, were kindly provided by the NIH (Rai et al, Journal of Medical Chemistry 2012) and were resuspended in DMSO. Hydrogen peroxide used for treatments was purchased from Sigma. Cisplatin (Sigma) was freshly resuspended in dimethylformamide before each use, while hydrogen peroxide was quantified each time before treatments. Cadmium Chloride (Sigma) was dissolved in sterile distilled water at a concentration of 1M and stored at -20°C.

5.3 Cell treatments

Ape1 inhibitors were used at the indicated doses, with or without genotoxic agents. For Western blot analysis, total cell extracts were obtained starting from $1*10^6$ cells prepared upon 24h of treatments if not differently indicated, and then analyzed through Western Blot. To perform viability assay or cell proliferation analysis upon 24h of treatment were used respectively $5*10^3$ and $3*10^5$ cells: three different wells were used for each condition of treatments.

5.4 Preparation of total cell extracts

Whole cell extracts (WCE) was prepared in the same way for all Western Blot analysis. Cells were harvested by trypsinization and centrifuged at 250 x g for 5 minutes at 4°C. Supernatant was removed and the pellet was washed once with ice-cold phosphate-buffered saline (PBS) and then centrifuged again as described before and removing the PBS. Cell pellet was resuspended in lysis buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% [wt/vol] Triton X-100 supplemented with 1X protease inhibitor cocktail (Sigma), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, and 1 mM Na_3VO_4, at a cell density of 10^7 cells/ml and rotated for 30 min at 4°C. After centrifugation at 12,000 x g for 10 min at 4°C, the supernatant was collected as total cell lysate. The protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

5.5 Western blotting and antibodies

Western blotting procedures were carried out using 30μg of total cell extract to detect all proteins. Protein extracts were electrophoresed onto a 12% SDS-PAGE and then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were saturated by incubation at 4°C overnight with 5% (wt/vol) nonfat dry milk in PBS-0.1% (wt/vol) Tween 20. Each filter was incubated with the specific antibody, notably for Polδ (ab10362; Abcam, Cambridge, MA), XRCC1 (MA1-12640; Thermo Scientific, Milan, Italy), Polβ (ab26343, Abcam), *p53* (sc-126; Santa Cruz Biotechnology, Santa Cruz, CA) and the monoclonal anti-Ape1 (211) antibody. Data normalization was performed by using a monoclonal anti-tubulin (Sigma) or anti-actin (Sigma) antibody. Detection was performed with the Odyssey Infrared imaging system (LI-COR Biosciences, Lincoln, NE). The protein bands were quantitated by densitometry, where the band intensity ratio of the treated group over the control group was calculated.

5.6 Viability assays and cell proliferation

Cells were seeded onto 96-well plates and treated for 24 hours with the indicated doses of the toxic agent with or without the specific Ape1 inhibitor reported

concentration. Cell viability was evaluated adding the 3 (4 5 dimethylthiazol 2 yl) 5 (3 carboxymethoxyphenyl) 2 (4 sulfophenyl) 2H-tetrazolium salt (MTS) solution to each well, depending on the mechanism reported in the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Results were obtained measuring absorbance at 240nm upon 1h and 30' of incubation at 37°C. for read was used a multiwall plate reader and values were standardized to wells containing media alone.
Cell proliferation was evaluated by trypan blue exclusion test. Trypan blue-excluding cells were collected from a 24-well plate and counted in a hemocytometer, in four randomly selected fields then averaged. Then the values obtained were reported in percentage in relation to the untreated one.

5.7 Gene expression analysis through Q-PCR

After treatment or silencing, total RNA from cell lines was extracted with the SV Total RNA isolation System kit (Promega). One microgram of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. qRT-PCR was performed with a CFX96 Real-Time System (Bio-Rad) using iQ™ SYBR® Green Supermix (Bio-Rad). The CFX Manager$_{TM}$ Software (BioRad) was used to define the expression of the following genes, using the indicated primers:

GENES	PRIMERS
Polδ	For 5'-GCTCCGCTCCTACACGCTCAA-3'
	Rev 5'-GTCTGGTCGTTCCCATTCTGC-3'
Pol β	For 5'-AGTACACCATCCGTCCCTTG-3'
	Rev 5'-AAAGATGTCTTTTTCACTATCCACTG-3'
XRCC1	For 5'-CTGGGACCGGGTCAAAAT-3'
	Rev 5'-CAAGCCAAAGGGGGAGTC-3'
APE1	For 5'- CCTGGACTCTCTCATCAATACTGG -3'
	Rev 5'- AGTCAAATTCAGCCACAATCACC -3'
GAPDH	For 5'- CCCTTCATTGACCTCAACTACATG -3'
	Rev 5'- TGGGATTTCCATTGATGACAAGC -3'
HPRT	For 5'- AGACTTTGCTTTCCTTGGTCAGG -3'
	Rev 5'- GTCTGGCTTATATCCAACACTTCG -3'

GAPDH and HPRT were used as internal controls to normalize samples.

5.8 Immuno-fluorescence and confocal microscopy

For immuno-fluorescence analyses, cells grown on glass coverslips were fixed with 4% paraformaldehyde for 20 minutes and extracted for 5 minutes in PBS/Triton X-100 0.1%. Cells were then saturated with 10% fetal bovine serum in TBS/Tween 20 0.1% and incubated over night with primary antibodies. AlexaFluor®488-conjugated secondary antibodies (Jackson ImmunoResearch) was used for detection. Cells were visualized through a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems) equipped with a 488-nm argon laser, a 543-nm HeNe laser, and a 63X oil immersion objective (HCX PL 11 APO CS 63X/1.32-0.60 – Leica). Data were acquired at room temperature (23°C) using the integrated Leica Confocal Software package; multi-color images were captured through sequential scanning.

5.9 Statistical analyses

Statistical analyses were performed by using the Student's t test. Either $\rho<0.05$ or $\rho<0.001$ were considered as statistically significant.

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7 Papers published during the PhD course (2012-2014)

Antoniali G., Lirussi L., D'Ambrosio C., Dal Piaz F., Vascotto C., Casarano E., Marasco D., Scaloni A., Fogolari F. and Tell G.
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SIRT1 gene expression upon genotoxic damage is regulated by APE1 through nCaRE-promoter elements

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ABSTRACT

The Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein contributing to genome stability, through the repair of DNA lesions via BER pathway and it also plays a role in gene expression regulation and in RNA metabolism. Another poorly characterized function is associated to its ability to bind to negative calcium responsive elements (nCaRE) of some gene promoters. The occurrence of many functional nCaRE sequences, regulating gene transcription, could be envisioned since their conservation within ALU repeats. Bioinformatic analyses, looking for functional nCaRE sequences within the human genome, resulted in a list of 57 genes, whose expression is potentially regulated by APE1. We focused on the SIRT1 deacetylase due to its involvement in cell stress, including senescence, apoptosis, tumorigenesis and its role in the deacetylation of APE1 after genotoxic stress. The human SIRT1 promoter presents two nCaRE elements stably bound by APE1 through its N-terminus. We demonstrate that APE1 is part of a multi-protein complex including hOGG1, Ku70 and RNA Pol II, playing a role in the regulatory function on the SIRT1 gene during early response to oxidative stress. These findings provide new insights in the comprehension of the role of nCaRE sequences in the transcriptional regulation of mammalian genes.

INTRODUCTION

The Apurinic/apyrimidinic endonuclease 1 (APE1), also known as Redox effector factor-1 (Ref-1), is a multifunctional and essential protein in mammals. It plays a vital role during the cellular response to oxidative stress (Fung *et al.*, 2005) and contributes to the maintenance of genome integrity (Tell *et al.*, 2005; Tell *et al.*, 2009; Tell *et al.*, 2010a). As AP-endonuclease, APE1 is involved in the base excision repair (BER) pathway that copes with DNA damages induced by oxidative and alkylating agents, including chemotherapeutics (Chen *et al.*, 2005). APE1 also has transcriptional regulatory activity, modulating gene expression through a redox-based co-activating function on several transcription factors involved in cancer promotion and progression (Huang *et al.*, 1993; Tell *et al.*, 1998; Gaiddon *et al.*, 1999). These two major APE1 activities are independent and located in distinct protein domains. The N-terminal portion of the protein is devoted to the transcriptional co-activating function; while, the C-terminal domain exerts the endonuclease activity on DNA abasic sites (Xanthoudakis *et al.*, 1996; Tell *et al.*, 2005). The latter domain is highly conserved; conversely, the N-terminal region presents wider variability among different organisms, being more conserved in mammals suggesting a recent acquisition during evolution (Georgiadis *et al.*, 2008; Fantini *et al.*, 2010; Poletto *et al.*, 2013). Through its N-terminal portion APE1 also interacts with different proteins involved in ribosome biogenesis, pre-mRNA maturation/splicing and ribonucleotide catabolism, highlighting an unexpected role of APE1 in RNA metabolism (Vascotto *et al.*, 2009b; Tell *et al.*, 2010b), as also shown by two independent studies regarding the ability of APE1 in cleaving abasic RNA *in vitro* and *in vivo* (Berquist *et al.*, 2008; Barnes *et al.*, 2009). Accordingly, APE1 has been proposed as a main candidate factor in the abasic RNA cleansing process, explaining some of the activities exerted by APE1 on gene expression through post-transcriptional mechanisms (Tell *et al.*, 2010b).

Another interesting, yet poorly characterized aspect of APE1 transcriptional activity is represented by its role as a component of a *trans*-acting complex, which acts as Ca^{2+}-dependent repressor of the parathyroid hormone (PTH) gene by binding the negative calcium responsive elements (nCaRE) in its promoter region (Okazaki *et al.*, 1991). In particular, an increase in extracellular Ca^{2+} concentration has been shown to inhibit PTH expression through a mechanism involving APE1 binding to two nCaRE elements, nCaRE-A and nCaRE-B (Yamamoto *et al.*, 1989). This observation was further extended to the promoter region of renin (Fuchs *et al.*, 2003), Bax (Bhattacharyya *et al.*, 2009) and APE1 itself (Izumi *et al.*, 1996); the latter case represents the first example of such a negative regulatory mechanism for a DNA repair enzyme. Subsequently, further experiments have demonstrated that APE1 requires other factors, such as heterogeneous ribonucleoprotein L (hnRNPL) (Kuninger *et al.*, 2002), Ku antigen (KuAg) (Chung *et al.*, 1996) and PARP-1 (Bhattacharyya *et al.*, 2009) to stably bind nCaRE elements.

nCaRE-B sequences are located within ALU repeats (McHaffie *et al.*, 1995;Shankar *et al.*, 2004), therefore, since ALU elements are transposable elements that occupy at least one tenth of the expressed human genome, many other functional nCaRE-B sequences could exist and play a role in the transcriptional regulation of genes. However, at present, specific information concerning an accurate number, the identity of genes containing these sequences within their own promoter, and how these elements play an active biological function are still evanescent. Thus, the quest for functional nCaRE-B sequences on human genome would identify new potential genes whose expression may be regulated by APE1 through nCaRE binding.

The present work is devoted to address this issue and is focused on the characterization of the molecular mechanisms responsible for APE1 binding to nCaRE-B sequences on SIRT1 promoter. Bioinformatic analyses of human gene expression data obtained upon APE1 knock-down in cells (Vascotto *et al.*, 2009a), revealed the presence of multiple nCaRE-B sequences in genes deregulated upon APE1 silencing and conserved in mouse genome. Among these, we studied those two present in the human deacetylase sirtuin 1 (SIRT1) gene promoter and their involvement in the corresponding gene transcription. SIRT1 is a deacetylase participating in cell growth, adaptation to caloric restriction, apoptosis, tumorigenesis (Gorospe *et al.*, 2008; Kim *et al.*, 2008) and playing

also a role in cell response to genotoxic agents through the deacetylation of APE1 (Yamamori *et al.*, 2010). Altogether, our data underline the importance of APE1 during the transcriptional initiation process, in positively promoting transcription of genes under genotoxic conditions.

RESULTS

Bioinformatic searching for nCaRE sequence-containing genes reveals SIRT1 gene as a novel candidate target of APE1 regulation

Bioinformatic analysis for the systematic retrieval of functional nCaRE-B sequences in the human genome was carried out filtering through biological data coming from gene expression profile of HeLa cells knocked down for APE1 (Vascotto *et al.*, 2009a). Here, we developed a method that integrates different approaches aiming to address the problem on a whole genome scale while minimizing the number of false positives. To this purpose, classical DNA pattern matching studies were integrated with independent information on gene regulation. We used three main sources for data filtering: i) functional annotation data collected in GO (Gene Ontology); ii) gene expression data derived from the microarray profile of APE1 knock-down HeLa cells (Vascotto *et al.*, 2009a); iii) human-mouse gene sequence comparisons. In fact, both expression data and functional annotation database are known to provide a wealth of information about co-regulation. This is of particular interest, since co-regulated genes likely share similar transcriptional regulatory mechanisms. At the same time, comparison with orthologous gene promoters highlighted sequences retained during evolution, whose conservation suggests their potential functionality. As a final result, we obtained a set of genes that passed the previously mentioned filters and might be considered *bona fide* as candidates co-regulated through nCaRE-B sequences (Supplemental Figure 1).

Experimentally, we collected the 6000 bp upstream regions of all human and mouse protein coding genes and then analysed for the presence of nCaRE-B elements using Glsearch as program for local alignment (Pearson, 2000). 8724 human genes were found to contain one or more nCaRE-B matches within their promoters; similarly, 2173 matches were retrieved in the mouse genome. We then cross-checked candidate genes with gene expression data obtained from APE1 knock-down cells (Vascotto *et al.*, 2009a), thus verifying some evidences of co-expression between genes carrying nCaRE-B elements. Through this analysis, we identified 384 common genes in the two datasets (Figure 1A). Then, we considered the GO annotations of these 384 genes, searching for statistically significant common annotations. We observed a strong over-representation of terms related to RNA processing and metabolism, in accordance to our previous studies (Vascotto *et al.*, 2009a). All the significant associations between genes and GO terms are reported in Supplemental Table S1. Finally, we applied the "phylogenetic footprinting filter" that evaluates whether the significant fraction of the genes, obtained through the GO filter, shares homologous genes, containing nCaRE-B related sequences in the upstream region, with the mouse gene dataset. As a final result, we extracted 57 genes that may be considered *bona fide* candidates bearing the putative nCaRE-B sequences within their regulatory elements (Supplemental Table S2). We performed a functional enrichment analysis to unveil if the 57 genes found are involved in common biological processes. This examination showed that candidate genes were associated with processes related to gene expression, activation or increment of the extent of transcription from an RNA polymerase II promoter (Figure 1B and Supplemental Table S3). Among these 57 genes, several are involved with DNA repair process and cellular response to external stimuli and DNA damage, e.g. SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4), sirtuin 1 (SIRT1), valosin-containing protein (VCP), multiple endocrine neoplasia I (MEN1), structural maintenance of chromosomes protein 6 (SMC6), early growth response 1 (EGR-1) and APE1 itself. Particular attention was focused on SIRT1, a NAD-dependent histone deacetylase belonging to the class III of the sirtuin family, based on the recent demonstration of a functional involvement of this enzyme in the deacetylation of some K residues in the N-terminal region of APE1 (Yamamori *et al.*, 2010; Lirussi *et al.*, 2012). The latter information and the data derived from our bioinformatic analysis, led us to hypothesize the existence of an auto-regulatory loop between APE1 and SIRT1.

APE1 binds the nCaRE-B sequences present in the human SIRT1 promoter

In order to prove the functional relevance of the nCaRE-B sequences identified in the human SIRT1 promoter, we first tested *in vitro* the ability of APE1 to specifically bind to these elements. Thus, we performed EMSA analyses using different APE1 recombinant proteins two double stranded (ds) oligonucleotides containing the SIRT1 nCaRE-B elements corresponding to the sequences found at -2701 (SIRT1-A) and -1754 bp (SIRT1-B) from the transcription start site (TSS), respectively (Figure 2A). Full length human wild type APE1 ($APE1^{WT}$), the N-terminal APE1 deletion mutant ($APE1^{N\Delta33}$) and the orthologous APE1 from zebrafish (zAPE1) expressed in *E. coli* were used to this purpose. As clearly demonstrated by the EMSA analyses, only the $APE1^{WT}$ protein was able to stably bind both the SIRT1 nCaRE-B sequences (Figure 2B, lanes 2 and 6), while a complete absence of retarded complex was observed in the case of the truncated $APE1^{N\Delta33}$ form (Figure 2B, lanes 3 and 7). These findings underlined the importance of the first 33 amino acids present at APE1 N-terminus for the proper binding of the protein to nCaRE-B sequences. A similar poor DNA binding activity was also evident in the case of zAPE1 (Figure 2B, lanes 4 and 8), which bears a non-related N-terminal domain (Fantini *et al.*, 2010). All together, these results suggest that the phylogenetically evolved N-terminal domain is essential for a stable interaction between APE1 and the nCaRE-B sequences; in this context, it is conceivable that K residues present within this region, and acquired during phylogenetic evolution in mammals (Georgiadis *et al.*, 2008; Fantini *et al.*, 2010; Poletto *et al.*, 2013), may play a major role in protein binding to these DNA elements.

We then estimated the affinity of APE1 for SIRT1 nCaRE-B sequences through SPR analysis (Figure 2C). Biotinylated versions of the nCaRE-B (SIRT1-B) or polyT sequences were immobilized onto a streptavidin chip to be used as ligands in SPR assays. $APE1^{WT}$ and $APE1^{N\Delta33}$ were then analyzed for their DNA binding activity. When testing $APE1^{WT}$ as analyte, a K_D value of 3.90±0.08 μM was measured (Poletto *et al.*, 2013); corresponding kinetic parameters are shown in Table 1. Conversely, when using $APE1^{N\Delta33}$, we did not observe any SPR signal variation (data not shown), in accordance with the EMSA analysis (Figure 2B). This result confirmed that the APE1 region 1-33 is essential for a stable interaction with these DNA elements. As DNA-repair enzyme APE1 has an intrinsic ability to bind DNA in a sequence-independent fashion, and independent observations clearly pointed to a role of the nucleic acid secondary structure in positively modulating this activity (Poletto *et al.*, 2013), we also evaluated the protein capacity to bind a single-stranded 24-mer oligo-dT (here called polyT). A K_D value of 308±3 μM was measured in the case of $APE1^{WT}$, while no binding was observed for $APE1^{N\Delta33}$ (Table 1 and Supplemental Figure S2), in agreement with EMSA analysis. These experiments indicated that APE1 poorly recognizes an oligonucleotide formed by stretch of thymidines, having a non-structured conformation, thus confirming that this protein may bind to DNA with different affinities and that the oligo-dT sequence may be used in EMSA analysis as non-specific competitor.

Our EMSA and SPR analyses on APE1 binding activity to the SIRT1 nCaRE-B sequences showed a low affinity for recombinant APE1; therefore, we tested whether additional factors present in nuclear extracts of cells may increase the protein binding affinity to its nucleotide target. To this aim, further EMSA analyses were performed on HeLa cells nuclear fractions, confirming that a nuclear activity able to specifically bind nCaRE-B sequences was indeed present (Figure 2D). The high affinity complex measured even using much lower amounts of APE1 (0.63 pmol, as estimated from (Lirussi *et al.*, 2012)) with respect to that obtained with the recombinant purified protein alone (10 pmol) (Figure 2B), suggested that additional factors are required for an efficient APE1 binding to SIRT1 nCaRE-B sequences. To demonstrate the presence of APE1 in the retarded complex observed in Figure 2D, we used nuclear extracts obtained from a HeLa lines (CL.3) where endogenous APE1 protein expression has been previously knocked-down through stable shRNA transfection (Vascotto *et al.*, 2009a). As apparent from lane 3 in Figure 2D, an overall reduction of the intensity (almost 70% with respect to lane 2) of the retarded nCaRE-bound complex was observed, similarly to what occurred also for the clone expressing $APE1^{N\Delta33}$ (lane 5) (almost 50% with respect to lane 2). Nuclear extracts obtained from HeLa cells reconstituted with ectopic

APE1^{WT} (lane 4) showed the same amount of the bound complex as the control cell clone expressing a scrambled shRNA vector (indicated as SCR-1) (recovery of almost 80% with respect to lane 2). Pre-incubation of the nuclear extract from the SCR-1 clone with an anti-APE1 antibody resulted in the formation of a super-shifted complex (Figure 2E, lane 3); this complex was absent when a non-related antibody was used (lane 6), clearly demonstrating that APE1, present in the nuclear cell extract, is involved in the recognition of the nCaRE-B elements of the SIRT1 promoter. The reduced intensity of the retarded complex band upon APE1 silencing or in the reconstituted cells expressing APE1$^{N\Delta33}$ (Figure 2D), as well as the lower apparent electrophoretic mobility of the protein-DNA retarded complex when using nuclear extracts in place of the recombinant purified protein (Figure 2B), suggest that APE1 may be part of a multi-protein complex. Since Ku70 antigen protein was already demonstrated to bind nCaRE-A sequences in complex with APE1 (Chung *et al.*, 1996), we incubated the HeLa nuclear extract with an antibody against the Ku70 antigen; reaction was then subjected to EMSA analysis. The formation of a super-shifted complex was evident (Figure 2E, lane 4). Concurrent presence of APE1 and Ku70 in the same retarded complex was confirmed by performing a simultaneous pre-incubation with antibodies against these proteins (Figure 2E, lane 5). The presence of Ku70 in the complex with APE1 was also corroborated by additional EMSA analysis performed with the purified recombinant protein attesting that, when present alone, Ku70 is not able to stably bind the SIRT1 nCaRE-B sequence *per se.* On the contrary, when concomitantly incubated with APE1, it significantly enhances its DNA binding activity to SIRT1 nCaRE-B element (Supplemental Figure 3 compare lanes 2-5 and 8-10). Data not shown demonstrated that this stimulatory activity on APE1 binding required the interaction through the APE1 33N-terminal domain. Overall, these data demonstrated that APE1 must be part of a multi-protein complex containing Ku70 to elicit its high-affinity binding potential to the nCaRE-B sequences present on SIRT1 promoter.

Topology of the APE1-nCaRE complex.

nCaRE-B element consists of a palindromic sequence that can possibly fold into self-complementary hairpins (Figure 3A, left panel). *In silico* analyses performed with mFold program were suggestive of the capacity of these elements to fold into cruciform-like structures. To evaluate whether SIRT1 nCaRE-B sequences can fold into cruciform duplexes and to specifically assess if APE1 binding to this element may depend on such secondary structures, we performed footprinting analyses by using the T7 endonuclease I (Figure 3A, right panel). This protein is a structure-sensitive enzyme that specifically recognizes conformationally-branched DNA and Holliday structures or junctions (Parkinson *et al.*, 1997; Déclais *et al.*, 2003; Fan *et al.*, 2006). Footprinting data supported the hypothesis of the existence of a secondary structure for the nCaRE-B sequences (Figure 3A and Supplemental Figure S4). In particular, our experiments showed the predominant occurrence of two bands (10 and 19 nt in length) in the digest, which corresponded to a cutting site close to the predicted loop and immediately adjacent to the predicted stem (Figure 3A, arrows). Interestingly, pre-incubation of the SIRT1 nCaRE-B oligonucleotide with APE1 impairs T7 endonuclease digestion (Figure 3A, lane 4). Consistent with these observations, EMSA analysis demonstrated that digestion of the SIRT1 nCaRE-B oligonucleotide with T7 endonuclease affects APE1 binding to this element but conversely, when APE1 was first incubated with the nCaRE-B probe and then digested with T7 endonuclease, the binding was not affected. These data were suggestive for a protective role of APE1 to T7 endonuclease action, strongly supporting the hypothesis that these proteins may compete for the same binding site on the nCaRE-B sequences (Figure 3B).

The first 33 N-terminal amino acids of APE1 are required for protein binding to nCaRE sequences (Figure 2, A and D). In order to investigate the role of this protein portion on APE1 ability of binding to these DNA sequences, the APE1-nCaRE complex was subjected to combined limited proteolysis-mass spectrometry experiments, according to previous studies realizing a differential probing of accessible amino acids in isolated and complexed proteins (Figure 3C and

Supplemental Table S4) (Scaloni *et al.*, 1998; Renzone *et al.*, 2007). When limited proteolysis experiments were carried out on recombinant APE1 protein in the absence or presence of its target nCaRE oligonucleotide, we obtained differential peptide maps, which we exploited to infer the residues present at the complex interface (Scaloni *et al.*, 1999). Interpretation of the results was based on the concept of "shielding" from proteolytic attack. In particular, limited proteolysis experiments were carried out on a time-course basis on isolated APE1, protein complexed with SIRT1 nCaRE-B oligonucleotide and protein complexed with PTH nCaRE-B oligonucleotide (Okazaki *et al.*, 1992); the latter was used as control. Data interpretation was rationalized according to the X-ray crystallographic APE1 structures reported so far (Gorman *et al.*, 1997; Beernink *et al.*, 2001).

Panels *I* and *IV* of Figure 3C show the time-course LC-ESI-MS analysis of the endoprotease AspN digestion on isolated APE1, as carried out by using an enzyme/substrate of 1:500 w/w. Under these experimental conditions, APE1 remained partly undigested, showing that its native conformation is susceptible to proteolysis at a unique, likely flexible site. After 5 min, only the peptide pair 1-17 and 18-321 was detected (Figure 3C, *panel I*), and no further fragments were released after 60 (Figure 3C, *panel IV*) and 120 min (data not shown) (Supplemental Table S4). As previously reported (Scaloni *et al.*, 1999), the definition of the primary cleavage sites was inferred by the identification of the complementary peptides released by a single proteolytic event occurring on the intact protein. According to these criteria, non-complexed APE1 was preferentially cleaved at D18 present within the unstructured protein N-terminal domain. When the APE1-SIRT1 nCaRE-B oligonucleotide complex was analyzed under the same conditions, no proteolytic fragments resulting from D18 cleavage were released even after 60 min (Figure 3C, *panel II* and *V*) (Supplemental Table S4), demonstrating a tighter conformation of the complex and a DNA-shielding effect for D18. A very reduced digestion at this site was observed only after incubation for 120 min (data not shown). A similar condition was verified also for the APE1-PTH nCaRE-B oligonucleotide complex, which showed absent or practically negligible proteolysis at D18 after 5 and 60 min, respectively (Figure 3C, panel *III* and *VI*). These results clearly demonstrate that after APE1 interaction with DNA, D18 is no longer accessible to the protease action. Thus, it may be hypothesized that this residue occurs at the protein-nCaRE oligonucleotide complex interface.

The different accessibility of the basic residues in isolated APE1 and in its complex with nCaRE-B oligonucleotides was similarly probed with trypsin, used at 1:5000 w/w enzyme/substrate (Supplemental Table S4). LC-ESI-MS analysis of the proteolytic fragments released from the isolated protein led to the identification of the complementary peptide pairs 1-9, 10-321 and 1-10, 11-321 already after 5 min of reaction, which were generated by single hydrolytic events at K9 and K10, respectively. Additional products resulting from fragment subdigestions were also observed, but they did not provide information on further accessible amino acids. These results confirmed that APE1 N-terminal portion is very flexible and highly exposed to the protease action. Conversely, appreciable hydrolysis of recombinant APE1 following protein interaction with SIRT1 nCaRE-B or APE1-PTH nCaRE-B oligonucleotides was observed at K9 only after 60 min of reaction (Supplemental Table S4). Due to the nature of the proteolytic products observed, these experiments confirmed the masking effect of both oligonucleotides over the non-structured protein N-terminal region.

Further experiments were then carried out with broader-specificity proteases, such as chymotrypsin and elastase. In the first case, limited proteolysis of isolated APE1 (enzyme/substrate of 1:1000 w/w) generated only a complementary peptide pair, namely 1-114 and 115-321, which identified L114 as the primary cleavage site (Supplemental Table S4). Additional products resulting from fragment subdigestions were also observed, but they did not provide information on further accessible amino acids. According to X-ray crystallographic data, this residue is exposed on the molecular surface of the globular APE1 domain (Gorman *et al.*, 1997). No significant differences were observed on a time-course basis when peptide maps were characterized for both APE1-nCaRE oligonucleotide complexes (Supplemenatl Table S4). In fact, both peptides 1-114 and 115-321 were

detected within the APE1-SIRT1 nCaRE-B and APE1-PTH nCaRE-B oligonucleotide products after 5 and 15 min, respectively, similarly to the non-complexed protein sample. These data excluded any involvement of L114 in binding to the nCaRE-B elements and further confirmed the suitability of the strategy used here.

When elastase digestion of isolated APE1 was carried out using an enzyme/substrate of 1:1000 w/w, only two peptide pairs (1-12, 13-321, and 1-14, 15-321) were detected after 5 min of reaction, whereas an additional pair (1-20, 21-321) was observed after 15 min (Supplemental Table S4). These data demonstrated that non-complexed APE1 was preferentially cleaved at A12, A14 and L20. When the recombinant APE1-SIRT1 nCaRE-B oligonucleotide and APE1-PTH nCaRE-B oligonucleotide complexes were analyzed under the same conditions, different proteolytic patterns were obtained, which proved preferential cleavage site at L20, as demonstrated by the identification of the complementary peptides 1-20 and 21-321 after 30 min of reaction (Supplemental Table S4). These results clearly demonstrated that, following complex formation, A12 and A14 are no longer accessible to elastase, whereas L20 is still partially exposed to the protease action.

The overall results of the limited proteolysis experiments for recombinant APE1 are summarized in Supplemental Table S4, from which a number of general considerations can be driven concerning the native protein. Preferential cleavage sites in isolated APE1 gathered into a specific region of the protein, the most exposed segment being the unstructured N-terminal domain, which contained 6 hydrolysis sites (K6, K7, A9, A11, D15 and L17). A further hydrolyzed peptide bond was located within the globular APE1 domain (L111). Interestingly, no other cleavage sites were detected in other protein regions, although exposed on the molecular surface (Gorman *et al.*, 1997; Beernink *et al.*, 2001). After complex formation, a marked protection effect exerted by nCaRE-B oligonucleotides was observed, as demonstrated by the large decrease in the number of proteolytic sites present in the N-terminal region (from 6 to 1), thus confirming the involvement of this APE1 portion in binding to these DNA elements.

APE1 regulates SIRT1 expression at the promoter level

To determine whether the observations obtained *in vitro*, regarding APE1 binding to SIRT1 nCaRE-B sequences, have any relevance *in vivo*, we examined the APE1 occupancy of the nCaRE-B sequence in the SIRT1 promoter through ChIP analyses. To this purpose, we used HeLa cells co-transfected with a human SIRT1 promoter-containing plasmid (Yamamori *et al.*, 2010) and FLAG-tagged $APE1^{WT}$- or $APE1^{N\Delta33}$-expressing plasmids. The amount of immunoprecipitated SIRT1 promoter was significantly enriched in $APE1^{WT}$-transfected cells, when compared with that obtained from control cells transfected with the empty vector alone (Figure 4A). As expected, $APE1^{N\Delta33}$-transfected cells had a remarkable reduction, even though not complete, in the amount of immunoprecipitated SIRT1 promoter. A similar degree of reduction was also observed when ChIP analysis was performed by using a SIRT1 promoter bearing a mutated sequence within the nCaRE-B motif (Figure 4B), whose reduced specificity was previously assessed through SPR analysis (Table 1 and Supplemental Figure S2). These experiments revealed that the mutation introduced in the nCaRE-B sequence significantly reduced the APE1 binding to DNA, lowering the affinity of the complex by a 30-fold factor (K_D = 119 ± 3 μM). Competitive EMSA analyses were in agreement with these findings. In fact, addition of the unlabeled nCaRE ds oligonucleotide resulted in an almost complete elimination of bound complex formation. On the other hand, competition with the unlabeled mutant nCaRE oligo (nCaRE-mut) caused only a slight reduction of the nCaRE-binding complex, in agreement with ChIP and SPR data, and supporting the notion of a sequence-dependent binding specificity (data not shown). Altogether, these results confirmed our *in vitro* observations (Figure 2) and support the notion that, under basal conditions, APE1 is associated with the nCaRE-B sequence within the SIRT1 promoter also *in vivo*, possibly as part of a multiprotein complex.

We then checked the functional relevance of APE1 on SIRT1 promoter activation by performing promoter-reporter assays. HeLa cells were co-transfected with a luciferase reporter vector bearing the SIRT1 promoter and $APE1^{WT}$ FLAG-tagged vector to evaluate if APE1 binding

to SIRT1 promoter may play a role in SIRT1 transcriptional regulation (Figure 4C). SIRT1 promoter-reporter assays showed that there was a significant increase in the luciferase signal detected in the presence of APE1, compared to that of the promoter alone. We evaluated the effect of APE1 silencing on endogenous SIRT1 mRNA expression levels through an inducible shRNA knock-down strategy (Vascotto *et al.*, 2009a,b). Endogenous APE1 knockdown (CL.3) caused a significant reduction in the SIRT1 endogenous expression levels (Figure 4D), which was rescued in cells reconstituted with a siRNA resistant APE1 cDNA expression plasmid (WT). These data demonstrated the positive effect of APE1 on SIRT1 transcriptional activation in accordance with our previous observations on gene expression profiling analysis (Vascotto *et al.*, 2009a), where a reduced expression of SIRT1 in APE1 knocked-down HeLa cells was apparent.

Oxidative stress induces SIRT1 transcription via recruitment of BER enzymes

In order to better understand the transcriptional function exerted by APE1 through the binding to nCaRE-B sequences, we investigated if APE1's positive role on SIRT1 transcription could rely on its enzymatic activity on DNA. First, we observed that under basal conditions APE1 does not present endonuclease activity on the SIRT1 nCaRE-B sequence, as assessed through an oligonucleotide cleavage assay (Figure 5A). APE1 cleavage at any site on cruciform nCaRE-B sequence should lead to a site-specific single-stranded break that can be detected by the appearance of an extra fragment in the cleavage assay. Even using increasing amount of purified recombinant APE1 protein we did not detect any nuclease activity that, on the contrary, was readily visible when using a radiolabeled 26-mer ds oligonucleotide containing a tetrahydrofuran mimicking an AP site (here referred as THF) (Berquist *et al.*, 2008). Substitution of the guanine residue at position 12, within the predicted loop of the nCaRE-B sequence, with a THF residue, resulted instead in efficient APE1 cleavage activity on the nCaRE-B sequence. This activity depends on the APE1 catalytic function, since the catalytically inactive $APE1^{E96A}$ mutant (Izumi *et al.*, 1999) was unable to efficiently cleave the same substrate (Figure 5B).

Prompted by these findings, we hypothesized that the APE1 positive function we observed on SIRT1 promoter may be ascribed to APE1 catalytic activity on nCaRE-B sequences; this phenomenon can be exerted after specific stimuli, such as oxidative stress, which can lead to abasic site formation on nCaRE-B sequences (Amente *et al.*, 2010; Francia *et al.*, 2012). It is well known that SIRT1 expression and function are regulated by external stressors, including the exposure to genotoxic agents (Kim *et al.*, 2008),28,(Cohen *et al.*, 2004). We therefore measured the SIRT1 transcription after an oxidative stress condition, such as that generated by H_2O_2 exposure. First, we demonstrated that SIRT1 promoter activation was increased by H_2O_2 treatment in a concentration-dependent fashion (Figure 5C). Next, we examined the effect of APE1 silencing or re-expression on SIRT1 transcriptional activation in HeLa cell clones upon H_2O_2-treatment (Figure 5D and Supplemental Figure S5). We treated the control (SCR-1), the APE1-silenced (CL.3) and the $APE1^{WT}$ cell clones with 1 mM H_2O_2 for 1 h. SIRT1 mRNA levels were then evaluated by Q-PCR analysis and compared with untreated clones. Upon oxidative treatment, SIRT1 mRNA resulted significantly increased and, notably, this response was higher in the presence of $APE1^{WT}$ protein, whereas it was lower in the case of APE1 knocked-down expressing cells. The residual activation of SIRT1 mRNA expression, which was apparent also in APE1 knocked-down cells, might be ascribable to the presence of remaining endogenous APE1 protein and/or to the existence of further limiting factors as already speculated (Chung *et al.*, 1996; Kuninger *et al.*, 2002; Bhattacharyya *et al.*, 2009). We confirmed the general relevance of our model by testing further hypothetical APE1 target genes containing an nCaRE-B element in their promoters and resulted dysregulated in APE1-kd cell model (Figure 1 and Supplemental Table S2) (Vascotto *et al.*, 2009a). To this aim, the expression levels, upon H_2O_2-treatment, of the Early growth response protein 1 (EGR-1) and Eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1), in the HeLa cell inducible-kd (CL.3) and in the reconstituted cell model (WT) used here, were evaluated by Q-PCR analysis (Figure 5E). Similar data were obtained in the case of MMS treatment (Supplemental

Figure S8). These data, showing inducible expression of these genes dependent on APE1 expression similar to that observed in the case of SIRT1, were suggestive of a general mechanism of gene activation upon DNA damage that involves APE1 binding to nCaRE-B elements.

To find a relationship between SIRT1 transcription induced by oxidative stress and the APE1 regulatory activity on the nCaRE-B sequences located within the promoter, we studied the dynamics of oxidative repair enzymes recruitment on the SIRT1 nCaRE-B sequence at early time upon H_2O_2 treatment. DNA base oxidation determines the formation of 8-oxodG, which is recognized by the DNA glycosylase OGG1. This enzyme initiates the BER pathway by removing the 8-oxodG lesion, which is further processed by APE1 that cleaves the apurinic site. To assess the dynamics of occupation of the nCaRE-B sequence on the SIRT1 promoter by these enzymes upon oxidative stress, we performed a time-course ChIP analysis on the SIRT1 nCaRE-B sequence after 1 mM H_2O_2 treatment (Figure 5F and Supplemental Figure S6). We immunoprecipitated SIRT1 nCaRE-B sequence with antibodies against 8-oxodG, OGG1 and APE1. We observed that the signal of 8-oxodG reached its pleteau promptly, 10 min upon H_2O_2 treatment and subsequently decreased, concomitantly with the accumulation of OGG1, that is recruited immediately later (15 minutes), in accordance with the BER processes. The occupancy by APE1 follows the OGG1 recruitment, consequently. To demonstrate a direct link between DNA repair and SIRT1 transcriptional initiation, we check the assembly on SIRT1 nCaRE-B sequence of RNA polymerase II (RNPII). Under basal condition, we observed that RNPII was found with relatively low abundance on the SIRT1 nCaRE-B sequence; conversely, the polymerase was progressively recruited as soon as the oxidative stress began (15 min after H_2O_2 treatment). Successively (at 40 min after H_2O_2 addition), RNAPII was again recruited on the SIRT1 promoter. This oxidatively induced recruitment of RNPII to the SIRT1 promoter, in concert with the observation that APE1 immunoprecipitated with RNPII with an augmented interaction resembling approximately the kinetics observed during ChIP analysis, suggest that oxidative stress could be a trigger for SIRT1 transcriptional activation (Supplemental Figure S7). It could be envisioned a mechanism in which H_2O_2 determines an oxidation of the guanine at the SIRT1 nCaRE-B sequence, thus recruiting components of the base excision repair system: OGG1 and APE1, together with proteins involved in nCaRE-B binding such as Ku70. When recruited to the SIRT1 promoter, APE1 (through its endonuclease activity) produces nicks on the SIRT1 nCaRE-B sequence, thus favouring the DNA relaxation necessary for the formation of chromatin loops that bring RNAPII at the transcription start site (Figure 6); the latter enzyme in turn can initiate transcription.

Discussion

After its cloning by independent groups, at first as a DNA repair enzyme (Demple *et al.*, 1991; Robson *et al.*, 1991) and then as a redox co-activator protein (Xanthoudakis *et al.*, 1992), a number of articles have described the APE1 functions, elucidating its involvement in several biological contexts. As the main apurinic/apyrimidinic endonuclease in mammalian cells, APE1 is classically renowned for its essential function as a DNA repair enzyme in the BER pathway. Beside this crucial role in the maintenance of the genome stability, APE1 was recently demonstrated to be also involved in redox signaling and in the regulation of gene expression (Tell *et al.*, 2010a; Wilson *et al.*, 2010), supporting the notion that it is a multifunctional protein, with features that go beyond the classical activities of as a DNA-repair enzyme. Notably, its multifunctional nature ascribes APE1 as an ideal candidate protein that links together DNA damage sensing/repair and transcriptional regulation of genes during cell response to genotoxic damage. Among these non-canonical activities, another interesting APE1 function is represented by its ability to bind the nCaRE sequence of some gene promoters, thus acting as a transcriptional regulator. Okazaki's group was the first that identified two nCaRE sequences within the PTH gene promoter (i.e nCaRE-A and nCaRE-B) (Okazaki *et al.*, 1991). The presence of these elements was also described in the regulatory region of few other genes, such as the human APE1 (Izumi *et al.*, 1996), the rat atrial natriuretic polypeptide (Okazaki *et al.*, 1992), the human renin (Fuchs *et al.*, 2003) and the bax ones (Bhattacharyya *et al.*, 2009). Beside these few genes, no further evidences have been provided so far. However, since nCaRE-B elements are present within ALU repeats that are widely distributed throughout the expressed genome, it is expected that APE1 could potentially regulate the expression of a large number of genes. Here, we performed an unbiased investigation on the whole human genome, searching for putative genes whose transcription may be mediated through APE1 ability to bind nCaRE-B elements. In particular, although it has been described that nCaRE elements seem to be active also at downstream regions (Izumi *et al.*, 1996), here, we focused in particular on nCaRE-B elements present only on the upstream sequence of human genes; in a near future, we plan to extend this approach also to downstream and intron regions. Bioinformatic analyses revealed a number of genes potentially regulated by APE1, which are involved in several pathways related to gene expression (Figure 1). Among the 57 candidate genes retrieved from our bioinformatic analysis, we chose to study the human deacetylase sirtuin1 (SIRT1) that bears two nCaRE-B elements in its promoter. Remarkable interest in SIRT1 was due to recent articles showing that this deacetylase controls the acetylation status of APE1 K6-7 (Yamamori *et al.*, 2010) and K27-35, thus modulating the subnuclear distribution of this protein and coordinating its enzymatic functions in BER pathway (Lirussi *et al.*, 2012). Therefore, we hypothesized the existence of a possible autoregulatory loop that can be established between the two proteins: APE1 should modulate SIRT1 expression that, in turn, may regulate APE1 functions through deacetylation.

To investigate the APE1 transcriptional regulatory function on SIRT1 expression, we first examined APE1 ability to bind the nCaRE-B sequences found in the SIRT1 promoter. Through different *in vitro* approaches, we showed that APE1 is able to bind SIRT1 nCaRE-B sequences (Figures 2 and 3). In particular, through EMSA and SPR analyses and limited proteolysis experiments we originally demonstrated that the APE1 N-terminal domain is essential for the proper binding of these elements. The essential role of this protein domain in DNA-binding is remarkable, particularly if we consider the phylogenesis of the nCaRE-B elements and that of the APE1 N-terminal region. nCaRE-B sequences are present within ALU repeats, which belong to SINE (short interspersed nucleotide elements) family of repetitive sequences that originally derived from the reverse transposition of 7SL RNA. This event took place in the genome of an ancestor of Supraprimates (Kriegs *et al.*, 2007); therefore, these repetitive elements have been found exclusively in primates (Deininger *et al.*, 1981), scandentians (Nishihara *et al.*, 2002) and rodents (Krayev *et al.*, 1980), all members of the placental mammalian clade Supraprimates (Euarchontoglires) (Murphy *et al.*, 2001). Similarly, current information regarding the sequence homology of the APE1 N-terminal domain across species, have pointed out the recent phylogenetic

acquisition of this region. Sequence conservation of this domain is very high in mammals but almost absent in other organisms with the exception of *Danio rerio*, *Dyctostelium* and *Drosophila*. Accordingly, it could be envisioned that once ALU elements appeared in primates and were stabilized in their genomes, progressively losing their transcriptional potential, these organisms needed to evolve novel mechanisms to cope with the acquired RNA pol II regulatory sites present within ALU region. The concomitant acquisition of the APE1 N-terminal domain in mammals could explain new modulatory functions towards these DNA elements. The observation that specific K residues (i.e. K24-27) within this reduced APE1 portion seem to be required for the correct binding of nCaRE-B (data not shown) is in nice accordance with this hypothesis. The zebrafish homologous of APE1, which shares with the human protein less than 40% of the N-terminal aminoacidic sequence and that lacks two out of five K residues in this region, is indeed no longer able to stably bind these nCaRE-B sequences (Figure 2B) (Poletto *et al.*, 2013). Similar results were also obtained when using an human recombinant APE1 mutant protein bearing specific K to A multiple substitution at K27/K31/K32/K35, in which the positive charges at amino acid side chain has been removed to mimic a condition similar to that exerted by K acetylation (data not shown).

The APE1 N-terminal domain seems required for the stable binding to the SIRT1 nCaRE-B elements, even though it is not sufficient (Poletto *et al.*, 2013). EMSA analysis, performed with the nuclear extracts of HeLa cells expressing the deletion form of APE1, indeed demonstrated that APE1 is certainly part of a multiprotein complex, being not the limiting factor in the binding reaction. As already speculated by Okazaki and also reported in later works, the binding affinity observed for the multiprotein complex is higher with respect to that detected when using purified APE1 protein alone (Figure 2). This suggested that other factors are necessary and cooperate with APE1 to fully exert this function, confirming previous observations (Chung *et al.*, 1996; Kuninger *et al.*, 2002). The two subunits of Ku antigen (Ku70 and Ku80) were among the protein factors already described by Chung *et al*. to be involved in the specific binding of nCaRE-A sequences (Chung *et al.*, 1996). Here, we demonstrated that Ku70 binding is not exclusively limited to the nCaRE-A elements, since we identified this protein in the complex that binds to the nCaRE-B sequence of SIRT1. The Ku heterodimer is a main component of the non-homologous end-joining (NHEJ) pathway repairing DNA double-strand breaks (DSBs), which are generally produced upon extensive oxidative and IR damage to DNA (Lieber, 2010). The peculiar structure of Ku allows recognition and tight binding to DSBs, together with the recruitment of DNA-PKcs and other factors to form the active protein kinase complex DNA-PK that facilitates processing and ligation of the broken ends (Walker *et al.*, 2001; Postow, 2011). Its involvement in the nCaRE binding is not clear, but emerging evidences underline the biological role of its non-canonical functions (Adelmant *et al.*, 2012). We envisage that Ku70 association with nCaRE elements could further facilitate APE1 binding, especially after DNA damage, since we observed an increased interaction between the two proteins upon an oxidative stress condition (Supplemental Figure S7).

We also better characterized the topology of the APE1-nCaRE complex. The palindromic nature of nCaRE-B sequences was already described by Okazaki *et al*. Authors suggested the possible involvement of a dimeric nuclear protein in this process (Okazaki *et al.*, 1991). Here, we suggested that the SIRT1 nCaRE-B, due to its palindromic sequence, can potentially fold into a cruciform-like structure and APE1 binding activity toward these elements strongly relies on the secondary conformation adopted by the oligonucleotide, as already established for other DNA and RNA substrates (Figure 3B and Supplemental Figure S4) (Poletto *et al.*, 2013). Formation of similar cruciform-forming palindromic sequences has been already described in eukaryotic cells and their biological consequences has been related to different processes, including regulation of transcriptional events when present in close proximity of gene promoters (Pearson *et al.*, 1994; Shlyakhtenko *et al.*, 2000; Alvarez *et al.*, 2002; Cunningham *et al.*, 2003; Kurahashi *et al.*, 2004). We therefore hypothesize a similar mechanism for SIRT1 transcriptional regulation. However, the requirement of a specific recognition motif also cannot be excluded since mutations in the SIRT1

nCaRE-B sequence, determining only a partial disruption of the oligonucleotide secondary structure, did not affect totally APE1 binding activity *in vivo* (Figure 4B).

We further deepened into APE1 transcriptional function on SIRT1 promoter. We found that APE1 positively affects SIRT1 gene transcription. Although APE1 was implicated in the repression of PTH gene transcription in a Ca^{2+}-dependent fashion, we observed that APE1 overexpression activated the SIRT1 promoter (Figure 4C) apparently through a Ca^{2+}-independent mechanism. This unexpected positive function on the transcription of a nCaRE-containing promoter was also reported in other works where authors suggested that the role of the nCaRE sequences in the context of different promoters and cells conditions could affect nCaRE activity (Bhakat *et al.*, 2003; Fuchs *et al.*, 2003). Interestingly, we noticed that the positive effect exerted by APE1 was particularly pronounced during oxidative stress, through its binding to SIRT1 nCaRE-B sequences (Figure 5D). Treatment with H_2O_2 leads to an activation of the SIRT1 promoter that determines an increase of the corresponding transcription in an APE1-dependent fashion. This positive transcriptional effect was also observed when we looked at the expression of other genes that present nCaRE-B elements in their promoters (Figure 5E), thus corroborating the hypothesis of a general mechanism of SIRT1 activation upon DNA damage that involves functional activation of APE1. These findings are in line with previous data from Yamamori *et al*. demonstrating that the genotoxic insult augments SIRT1 expression and, therefore, its deacetylase activity on APE1 K6/7, favoring APE1 binding to XRCC1 (Yamamori *et al.*, 2010). Interestingly, these authors evidenced that a decrease of APE1 acetylation at later times after oxidative treatment is usually accompanied by SIRT1 up-regulation. All together, these findings are in accordance with a model of a positive autoregulatory loop between the two proteins. Thus, SIRT1 seems to be involved in a feedback mechanism that shuts off the cellular response mediated by APE1 acetylation (Yamamori *et al.*, 2010).

It has been suggested that DNA oxidation could trigger positive transcription in the context of Myc-mediated transcription through the involvement of BER enzymes, including APE1 (Perillo *et al.*, 2008; Gillespie *et al.*, 2010). Similarly, we speculated that the APE1 positive effect observed on SIRT1 transcription might depend on APE1 endonuclease activity over the nCaRE-B elements present within SIRT1 promoter. We here propose a model where oxidative-mediated DNA repair and gene transcription are linked together (Figure 6). During oxidative stress conditions, DNA oxidation determines the formation of 8-oxodeoxyguanine (8-oxodG) lesions, which are recognized and processed by enzymes of the BER pathway, including APE1. In our model, the oxidative burst is an early event essential for the formation of a productive transcription initiation complex, which relies on the initial recruitment of BER enzymes. The nicks introduced at the chromatin level by APE1, during 8-oxodG removal might promote the local relaxation required for the formation of chromatin loops moving closer the active form of RNA polymerase II to the TSS of the gene, as previously recruited by APE1 on nCaRE-B sequence, turning on the transcription. Our data, therefore, can be generalized into a regulatory model for all those genes that contain nCaRE-B elements. Accordingly, a new hypothesis can be proposed for the molecular activation of specific genes during early response to DNA damage. This model links together DNA-repair enzymes and transcriptional regulation effectors and may constitute a general model for explaining the adaptive cell response to oxidative stress involving gene regulation and DNA damage.

MATERIALS AND METHODS

Bioinformatic analysis

All human and mouse DNA sequences were retrieved from the Ensembl database (release 56) (http://www.ensembl.org/) by using a dedicated program written in Perl that collects entries from this archive. A sequence window that contains 5' genomic DNA of every gene coding for a protein was selected. This region extends from 6000 bp upstream and 1000 bp downstream of each transcriptional start site. Gene Ontology (GO) annotations were obtained from Ensembl database by using the data mining tool BioMart (http://www.ensembl.org/) (Spudich *et al.*, 2007). Human and mouse promoter regions were scanned for significant similarities to nCaRE-B by using Glsearch as program for local alignment (available in the Fasta3 program package) (Pearson, 2000). Glsearch was chosen because it calculates an alignment that is global in the query and local in the library. The following nCaRE-B sequences were used as query (Izumi *et al.*, 1996):

Name	Sequence (5' to 3')
nCaRE-B (PTH)	TTTTTGAGACAGGGTCTCACTCTG
nCaRE-B1 (APE1)	TTTTGAGACAGTCTCAGCTCTG
nCaRE-B2 (APE1)	TTTTGAGACAGAGTTTCACTCTTG

Alignments were computed with Altschul and Gish's statistical estimates, which are more suitable for searching of short query sequences (-z 3 option) (Altschul, 1991). We selected only those promoter genes that showed one or more matches for nCaRE-B sequences, allowing up to two mismatches in the case of human genome and up to three mismatches in the case of mouse one; in fact, in the latter case, most alignments were found with three mismatches. From mouse promoter genes that contained nCaRE-B elements, we retrieved only orthologous man/mouse genes, as obtained from the BioMart Ensembl database. For the microarray filter, we cross-checked human genes selected from alignment search with microarray data obtained from the gene expression profile of HeLa cells silenced for APE1 by RNA interference (Vascotto *et al.*, 2009a). GO filter identified co-regulated human genes, as determined by microarray analysis, studying the prevalence of their GO annotation terms. This analysis was obtained by using a Perl program kindly provided by Caselle and coworkers (Corà *et al.*, 2004), which performs an exact Fisher's test based on hypergeometric distribution to determine whether the term appears in the set significantly more often than what expected by chance. This program uses four different entries: i) a file containing the whole GO database structure (OBO version 1.2, available at http://www.geneontology.org/); ii) the list of genes from whole human genome; iii) a list of all genes with all the GO terms associated with them (as obtained from Ensemble-BioMart); iv) the set of genes to be tested. In general, a GO annotation term was considered to be significantly overexpressed when the corresponding *p*-value (not corrected for multiple testing) was lower than 1E-4. Phylogenetic footprinting analysis consisted in the last selection from significant data obtained from GO filter of that genes also present in the mouse orthologous dataset.

Gene annotations co-occurrence analysis

Gene identifiers corresponding to the list of 57 putative genes regulated by APE1 were submitted to GeneCodis (http://genecodis.cnb.csic.es/), a web-based tool for the ontological analysis (Carmona-Saez *et al.*, 2007; Nogales-Cadenas *et al.*, 2009; Tabas-Madrid *et al.*, 2012), selecting *Homo Sapiens* as the source for annotations and 'Biological Process' as the Gene Ontology category to perform the gene annotation co-occurrence analysis.

Cell culture and transient transfection experiments

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy), 100 U/ml penicillin, and 10 μg/ml

streptomycin sulfate. One day before transfection, cells were seeded in 10-cm plates at a density of 3×10^6 cells/plate. Cells were then transiently transfected with plasmids of interest by using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

Inducible APE1 knock-down and generation of APE1 knock-in cell lines

Inducible silencing of endogenous APE1 and reconstitution with mutant proteins in HeLa cell clones was performed as already described (Vascotto *et al.*, 2009a,b). For inducible shRNA experiments, doxycycline (1 μg/ml) (Sigma) was added to the cell culture medium and cells were grown for 10 days.

Plasmids and expression of recombinant proteins

Plasmid containing the human SIRT1 promoter was kindly provided by Dr. Irani, University of Pittsburgh, USA. This plasmid consists of a fragment of the human SIRT1 promoter (–1266 to +137 relative to transcription start site) cloned into the pGL4.1 firefly luciferase reporter vector (Promega) (Yamamori *et al.*, 2010). The human SIRT1 promoter carrying the mutation at nCaRE-B sequences was generated with a Site-Directed Mutagenesis Kit (Stratagene), using the following primers:
SIRT1-B mut for 5'-TCATCTAGGTTTTATTTATATATTTTTTTGCTAAGGAGCGTCGCTCTTGCTGCCCAGGCTGGTGTG-3' and SIRT1-B mut rev5'-CACACCAGCCTGGGCAGCAAGAGCGACGCTCCTTAGCAAAAAAATATATAAATAAAACCTAGATGA-3'.

Expression and purification of recombinant proteins from *E. coli* were performed as previously described (Vascotto *et al.*, 2009b; Fantini *et al.*, 2010).

Antibodies and Western blotting analysis

For Western blotting analyses, the indicated amounts of cell extracts were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 5% w/v non-fat dry milk in PBS containing 0.1% v/v Tween 20 and probed with the monoclonal anti-FLAG antibody (Sigma), the monoclonal anti-APE1 antibody (Vascotto *et al.*, 2009a), the monoclonal anti-Ku70 (sc-12729, Santa Cruz Biotechnology, Inc.) and the monoclonal anti-RNA polymerase II (Abcam); blots were developed by using the ECL enhanced chemiluminescence procedure (GE Healthcare) or Western Lightning Ultra (Perkin Elmer). Data normalization was performed by using a monoclonal anti-tubulin antibody (Sigma). Blots were quantified by using a Chemidoc XRS video densitometer (Bio-Rad).

Secondary structure predictions

Potential secondary structures for SIRT1-B nCaRE-B oligonucleotide were determined by using the mFold Web Server program available at (http://mfold.rna.albany.edu/?q=mfold). Structure predictions were run by setting the program parameters as close as possible to the conditions used in binding assays (i.e. 37°C and 50 mM monovalent cation).

Chromatin immunoprecipitation (ChIP) Analysis

ChIP assay was performed by using a protocol described previously (Lirussi *et al.*, 2012).

Preparation of nuclear cell extracts

Nuclear protein extracts were prepared as described earlier (Ziel *et al.*, 2005).

Electrophoretic Mobility Shift Assay (EMSA) analysis

APE1 binding to nucleic acids was assessed as already described (Fantini *et al.*, 2010), with some modifications. Briefly, the indicated amount of recombinant proteins or 5 µg of the reported nuclear extract were incubated at 37°C for 15 minutes with 250 pmol of unlabeled poly(dT), or 250 ng of sonicated salmon sperm DNA (Sigma). 2.5 pmol of ^{32}P-labeled double-stranded (ds) oligonucleotides were then added and incubated for additional 15 min and further separated onto a native 6% w/v polyacrylamide gel at 150 V, for 4 h. When performing super-shift assays, 5 µl of monoclonal anti-APE1 (Vascotto *et al.*, 2009a), anti-Ku-70 (sc-12729, Santa Cruz Biotechnology, Inc.) or anti-P2Y6 (Alomone Labs) were pre-incubated with HeLa nuclear extract from APE1^{SCR-1} clone at 4°C, for 3 h.

Oligonucleotides used for EMSA were the following:

Name	Sequence (5' to 3')
nCaRE-B SIRT1-A	For TTTTTGAGACAGAGTTTCACTCTTG
	Rev CAAGAGTGAAACTCTGTCTCAAAAA
nCaRE-B SIRT1-B	For TTTTTGAGACGGAGTTTCGCTCTTG
	Rev CAAGAGCGAAACTCCGTCTCAAAAA
Poly(dT)	For TTTTTTTTTTTTTTTTTTTTTTT

T7 endonuclease I footprinting

Footprinting analysis on SIRT1 nCaRE-B sequence were conducted using T7 endonuclease I. Briefly, 5'-^{32}P-end-labeled SIRT1-B nCaRE-B was digested with 5U of T7 endonuclease I at 37°C, for 1h. The reaction mixtures were then loaded and separated for 2h onto a denaturing 8M urea sequencing gel. After separation, the gel was incubated for 30' in a 10% methanol and 10% acetic acid solution for 30' and then wrapped in Saran wrap and exposed to film for autoradiograpy.

Determination of AP endonuclease activity

Determination of APE1 AP endonuclease activity was performed using an oligonucleotide cleavage assay, as described previously (Vascotto *et al.*, 2009b). The indicated amount of recombinant APE1 protein was incubated with a 5'-^{32}P-end-labeled 26 mer ds oligonucleotide containing a single tetrahydrofuranyl (here called THF) artificial AP site at position 14, which is cleaved to a 14-mer in the presence of AP endonuclease activity. Alternatively, a 5'-^{32}P-end-labeled ds SIRT1-B nCaRE-B oligonucleotide or a 5'-^{32}P-end-labeled ds nCaRE SIRT1-B oligonucleotide, bearing a single tetrahydrofuranyl residue at position 12 (bolded) 5'-TTTTTGAGACG**G**AGTTTCGCTCTTG -3' (Integrated DNA Technologies) were used.

Reporter assays

For reporter assay experiments, we used a human SIRT1 promoter plasmid allowing for promoter activity measurements upon luciferase assay, as already described (Yamamori *et al.*, 2010). To this purpose, 2.5x10^4 HeLa cells were seeded in a 96-well plate and co-transfected with 15 ng of human SIRT1 promoter, 0.3 ng of a constitutive renilla reporter plasmid and 75 ng of a vector expressing FLAG-tagged APE1 protein. When performing luciferase assays upon H_2O_2, cells were challenged with increasing amounts of H_2O_2 in serum-free medium for 1h, at 37°C, and then firefly and renilla luciferase activities were measured 24 h after the treatment by using the Dual-Glo Luciferase assay system (Promega), according to manufacturer's recommendations. Firefly activity was normalized to renilla activity to correct for differences in transfection efficiency. Results are from triplicate experiments.

Q-PCR

Total RNA from cell lines was extracted with the SV Total RNA isolation System kit (Promega). One microgram of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. qRT-PCR was performed with a CFX96 Real-Time System (Bio-Rad) using iQ™ SYBR® Green Supermix (Bio-Rad). Primer

sequences for human SIRT1 were those reported in (Yamamori *et al.*, 2010). Human GAPDH was used as internal control; sense, 5'- CCCTTCATTGACCTCAACTACATG -3'; antisense 5'- TGGGATTTCCATTGATGACAAGC -3'.

Surface Plasmon Resonance (SPR) analysis

Real time binding assays were performed on a Biacore 3000 SPR instrument (GE Healthcare). Biotinylated ds oligonucleotides were immobilized on a SA-chip at the desired level, as result of their injection at a concentration of 500 nM in HBS (20 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% v/v P20 surfactant, 0.1 mM tris(2-carboxyethyl)phosphine), at 10 μL/min as flow rate. Flow cell 2 contained 100 RU of poly(dT); flow cell 3 and 4 contained 77 and 60 RU of SIRT1-B nCaRE-B and SIRT1-B nCaRE-B mutated respectively, and flow cell 1 (with streptavidin) was left blank to be used as a reference surface. APE1 and its deletion mutant APE1$^{N\Delta 33}$ were serially diluted in running buffer to the indicated concentrations and injected at a flow rate of 20 μL/min for 4.5 min, at 20°C. Disruption of any complex that remained bound after a 3-min dissociation was achieved by using an injection of 1 M NaCl at 20 μL/min, for 1 min. BIAevaluation analysis package version 4.1 (GE Healthcare) was used to subtract blank signal, and to evaluate kinetic and dissociation constants. Kinetic parameters were estimated assuming a 1:1 binding model and using version 7 4.1 Evaluation Software (GE Healthcare). An affinity steady state model was applied to fit the RUmax data versus proteins concentrations and fitting was performed with GraphPad Prism v4.00 (Fantini *et al.*, 2010).

Limited proteolysis

Suitable experimental conditions were chosen by testing proteolysis with different enzyme/substrate values; no preventive removal of DNA was performed. Thus, limited proteolysis experiments on recombinant APE1 were conducted in 50 mM NH_4HCO_3, pH 7.5 (reaction buffer) at 37°C, by using an enzyme to substrate ratio ranging from 1:500 to 1:5000 w/w. Three identical aliquots of APE1 (500 pmol) were combined with reaction buffer or DNA nCaRE-B ds oligonucleotides (PTH and nCaRE SIRT1-B) (5:1 mol DNA/protein) dissolved in reaction buffer to generate samples (100 μl final volume each), which were incubated for 15 min at 37°C, before protease addition. After digestion starting, the extent of proteolysis was monitored on a time-course basis by sampling 10 μl of the mixture at time intervals ranging from 5 to 120 min. Reaction samples were immediately quenched with 5% formic acid and then frozen in dry-ice before LC-ESI-MS analysis.

LC-ESI-MS analysis

APE1 digests were analyzed with Q-TOF Premier mass spectrometer (Waters, Milford, MA) equipped with a nanospray source. Peptide mixtures were separated on an Atlantis C_{18} column (100 μm x 100 mm, 3 μm), using a linear gradient ranging from 30 to 60% acetonitrile in 1% formic acid, over a period of 50 min, at a flow rate of 800 nl/min. Spectra were acquired in the *m/z* 650-2500 range. Data were processed by using the MassLynx software (Waters). Mass calibration was performed by means of the multiply charged ions from horse heart myoglobin (Sigma). Depending on polypeptide size, mass values have been reported as monoisotopic or average values. Observed mass values were assigned to specific polypeptides by using the Paws software (Proteometrics Inc.), based on APE1 sequence and selectivity of the protease used for protein digestion.

Statistical analyses

Statistical analyses were performed by using the Microsoft Excel data analysis program for Student's t test analysis. $P < 0.05$ was considered as statistically significant.

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Figure Legends

Figure 1. Bioinformatic research of nCaRE sequences. (A) Results obtained from the application of the different filters. *Upper panel*, data derived from alignment research of nCaRE sequences on

human gene promoters and the subsequent cross-check with microarray data. *Bottom panel*, final results deriving from the combined Gene ontology and phylogenetic footprinting analyses. (B) Functional enrichment analysis of the 57 putative genes regulated by APE1 performed according to biological process. For simplicity, only the most representative functional categories are reported. The number of genes for each category is provided on the horizontal axis, together with the list of the first seventeen co-occurrence terms. Statistical significance belonging to each category is shown within each bar.

Figure 2. APE1 is part of a nuclear protein complex that binds to SIRT1 nCaRE sequence through its N-terminal domain. (A) *Upper panel*, schematic representation of two nCaRE-B sequences in human SIRT1 gene promoter located at -2701 bp (nCaRE SIRT1-A) and -1754 bp (nCaRE SIRT1-B) from SIRT1 transcriptional start site (TSS). *Bottom panel*, multiple sequence alignment of the two nCaRE sequences found in the human SIRT1 promoter and the nCaRE sequence found on the human PTH promoter (Okazaki *et al.*, 1991). Multiple alignments were performed using the software CLUSTALW2, available at (http://www.ebi.ac.uk/Tools/msa/clustalw2/). (B). EMSA analysis with ds oligonucleotides corresponding to nCaRE SIRT1-A and SIRT1-B sequence found in the human SIRT1 promoter that was challenged with $APE1^{WT}$, $APE1^{N\Delta33}$ and zAPE1. Reactions were performed with 2.5 pmol of oligonucleotide and 10 pmol of purified $APE1^{WT}$ (lanes 2, 6), $APE1^{N\Delta33}$ (lanes 3, 7) and zAPE1 (lanes 4, 8). Lane 1 and 5 represent probe alone; F shows the position of the free oligonucleotide probe. Specific APE1/nCaRE interaction is indicated by the arrow. Coomassie staining of the purified recombinant proteins challenged in EMSA is shown on the left. (C) SPR analysis on the hAPE1–nCaRE interaction. Recombinant hAPE1 and biotinylated nCaRE SIRT1-B (Table 1) was used as analyte and ligand, respectively. Plot of RUmax from each binding vs. hAPE1 concentrations (0.5-8 μM); data were fitted by non-linear regression analysis. (D) *Upper panel*, 2.5 pmol of ds oligonucleotide corresponding to nCaRE SIRT1-B were incubated with 5 μg of HeLa nuclear extract of different clones: control clone, $APE1^{SCR-1}$ (lane 2), clone silenced for APE1, $APE1^{CL.3}$ (lane 3), clones where the expression of APE1 is reconstituted with $APE1^{WT}$ (lane 4) or with $APE1^{N\Delta33}$ (lane 5). Lanes 1 corresponds to probe alone; F shows the position of the free oligonucleotide probe. Specific APE1/nCaRE interaction is indicated by the arrow. *Bottom panel*, Western blotting analysis with APE1 antibody of the nuclear cell extracts reveals the silencing of endogenous APE1 ($APE1^{CL.3}$), when compared with control clone transfected with the empty vector ($APE1^{SCR-1}$) and that expression the ectopic flagged $APE1^{WT}$ and $APE1^{N\Delta33}$. Tubulin was used as protein loading control. (E) 2.5 pmol of ds oligonucleotide corresponding to nCaRE SIRT1-B were incubated with HeLa nuclear extract from $APE1^{SCR-1}$ clone alone (lane 2) or pre-incubated with monoclonal antibody against APE1 (lane 3) or/and with an antibody against Ku-70 (lanes 4 and 5). Lane 6 corresponds to $APE1^{SCR-1}$ nuclear extract incubated with an aspecific antibody (α-P2Y6). Lane 1 is probe alone; F shows the position of the free oligonucleotide probe. Specific APE1/nCaRE interaction is indicated by the arrow. Asterisk indicates super-shift.

Figure 3. APE1 recognizes structured nCaRE sequences trough its N-terminal domain. (A) *Left*, predicted cruciform structure of nCaRE SIRT1-B double-stranded oligonucleotide as obtained by using mFold. Arrows indicated the cleavage site of T7 endonuclease I and the length of the products. *Right*, 5'-^{32}P-end-labeled nCaRE was pre-incubated (lane 4) or not (lane 3) with 35 pmol of APE1 recombinant protein for 15 min at 37°C in EMSA binding buffer, then 5U of T7 endonuclease I were added to each reaction. Pre-incubation of nCaRE with APE1 impairs T7 endonuclease digestion (lane 4). (B) EMSA analysis of APE1 binding to nCaRE sequence after digestion with T7 endonuclease or after pre-incubation with APE1 and subsequent digestion with T7 endonuclease. 2.5 pmol of ds oligonucleotide corresponding to nCaRE SIRT1-B were first digested with T7 endonuclease at 37°C, for 1 h, and subsequently incubated with increasing amounts of recombinant APE1 (10 or 30 pmol) at 37°C, for 15 min (lanes 7, 8). Alternatively, 2.5 pmol of nCaRE probe were first incubated with APE1 at 37°C, for 15 min, and sequentially

digested with T7 endonuclease at 37°C, for 1 h (lanes 9, 10). Lanes 1 is probe alone; 1st and 2nd indicate if APE1 incubation with the probe was performed temporally before (1st) or after (2nd) T7 digestion; F shows the position of the free oligonucleotide probe. F** indicates the T7 endonuclease-digested probe. Specific APE1/nCaRE interaction is indicated by the arrow. (C) Comparative limited proteolysis experiments on isolated or DNA-complexed APE1. Time-course analysis of simultaneous trials performed on non-complexed recombinant APE1 (I, IV), recombinant APE1-SIRT1 nCaRE-B complex (II, V) or recombinant APE1-PTH nCaRE complex (III, VI) are shown. LC-ESI-MS profiles from samples taken at 5 (I- III) and 60 min (IV-VI) are reported. Identified peptides are reported on the corresponding chromatographic peaks; 1-321 denotes the intact protein.

Figure 4. APE1 positively regulates SIRT1 expression at the promoter level. (A). *Upper panel*, schematic representation of the human SIRT1 promoter used for HeLa transfection which included the nearest nCaRE sequence from TSS (nCaRE SIRT1-B). *For* and *Rev* arrows indicated the position of the RT-PCR primer designed for the quantification of the human SIRT1 nCaRE sequence bound to APE1. *Middle panel*, ChIP assay for APE1-nCaRE sequence association. HeLa cells were co-trasfected with both human SIRT1 promoter and with vector expressing $APE1^{WT}$, $APE1^{N\Delta 33}$-FLAG tagged, or the empty vector as ChIP negative control. The values reported were calculated as fold percentage of the amount of immunoprecipitated nCaRE DNA relative to that present in total input chromatin. Data were further normalized to the amount of immunoprecipitated protein. *Bottom panel*, Western blotting analysis was performed on total cell extracts (input) and on immunoprecipitated material (IP) with specific antibody for FLAG and APE1. IB: immunoblot. The significance of sample average difference observed was estimated by Student's t test. * p-value < 0.05. (B) ChIP analysis on mutated human SIRT1 promoter. *Upper panel*, base composition of the nCaRE sequence of human SIRT1 promoter (-1754 bp from TSS) and of the the mutated sequence used for site-directed mutagenesis. Divergent sequences in the mutant nCaRE are bold. *Middle panel*, HeLa cells were co-transfected with empty vector or with vector expressing $APE1^{WT}$ and, alternatively, with wild type hSIRT1 promoter or hSIRT1 promoter carrying a mutation on its nCaRE-B sequence. The histogram represents the amount of hSIRT1 promoter sequence that was immunoprecipitated. Data are presented as percent of input and were normalized to the amount of APE1 immunoprecipitated, as evaluated by Western blotting analysis (*bottom panel*). The significance of sample average difference observed was estimated by Student's t test. * p-value < 0.05. (C) hSIRT1 promoter is activated in presence of APE1. *Upper panel*, reporter assay with hSIRT1 firefly reporter vector co-trasfected with $APE1^{WT}$ FLAG-tagged vector. Firefly luciferase activity observed was normalized to renilla luciferase activity. *Bottom panel*, Western blotting analysis for the normalization of protein levels. Tubulin protein level was used to normalize samples. (D) Analysis of SIRT1 mRNA level with Q-PCR in clones expressing $APE1^{WT}$ or APE1 silenced (CL.3) cells. *Upper panel*, data shown in the histogram are normalized to the amount of GPDH. The significance of sample average difference observed was estimated by Student's t test. A p-value < 0.001 was considered as statistically significant (*). *Bottom panel*, Western blotting analysis on protein extract of clones showing the suppression of endogenous APE1 expression, upon ten days of treatment with doxycycline. Tubulin protein level was used to normalize samples.

Figure 5. Recruitment of BER enzymes on the SIRT1 promoter. (A) APE1 endonuclease activity on ds nCaRE SIRT1-B radiolabeled oligonucleotide was tested by using an AP endonuclease activity assay. A radiolabeled ds THF-containing deoxyoligonulceotide (THF) was used as control. Reactions were performed with 2.5 pmol of each probe and increasing amounts (pmol) of recombinant $APE1^{WT}$ protein. The conversion of the radiolabeled THF-containing oligonucleotide substrate (S) to the shorter product (P) was evaluated on a denaturing 20% polyacrylamide gel. The corresponding gel image of the enzymatic reactions is shown. (B) APE1 AP endonuclease activity on 2.5 pmol of nCaRE SIRT1-B THF-containing probe incubated with increasing amounts of

recombinant APE1^{WT} protein or a catalytic inactive APE1 mutant (APE1^{E96A}). The conversion of the radiolabeled THF-containing oligonucleotide substrate (S) to the shorter product (P) was evaluated on a denaturing 20% polyacrylamide gel. The corresponding gel image of the enzymatic reactions is shown. (C) Reporter assay with HeLa cells transfected with hSIRT1 firefly reporter vector and challenged with increasing doses of H_2O_2, for 1h, as indicated. Firefly luciferase activity observed was normalized to renilla luciferase activity. A *p*-value < 0.001 was considered as statistically significant (*). (D) *Upper panel*, Q-PCR analysis of SIRT1 mRNA levels in clones expressing APE1^{WT} or APE1 silenced cells APE1$^{CL.3}$ after 1 mM H_2O_2 treatment, for 1 h. Data shown in the histogram are normalized to the amount of GAPDH. The significance of sample average difference observed was estimated by Student's t test. A *p*-value < 0.05 was considered as statistically significant (*). *Bottom panel*, Western blotting analysis on the protein extracts described in the upper panel. Tubulin protein level was used to normalize samples.(E) Q-PCR analysis of SIRT1, EGR-1 and EIF4EBP mRNA levels in APE1 silenced (APE1$^{CL.3}$) or expressing APE1^{WT} clones after 1 mM H_2O_2 treatment, for 1 h. Data shown in the histogram are reported as fold of activation after H_2O_2 treatment and normalized to the amount of GAPDH. The significance of sample average difference observed was estimated by Student's t test. A *p*-value < 0.05 was considered as statistically significant (*). (F) Histogram reports the trend of four independent ChIP analyses relative to the accumulation of 8-oxoGs, OGG1, APE1 and RNA polymerase II protein on the SIRT1 promoter after H_2O_2 treatment. HeLa cells were co-transfected with empty vector or with a vector expressing APE1^{WT} and hSIRT1 promoter and challenged with 1 mM H_2O_2, for different times (as reported). Data are presented as percent of input and were normalized to the quantity of DNA immunoprecipitated by α-tubulin (α-tub). See Fig. S4 for detailed information.

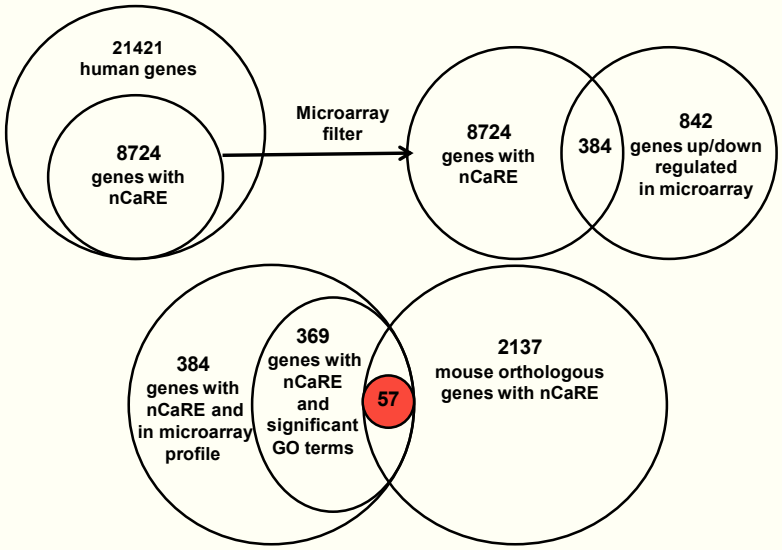
Figure 6. Mechanistic model for the role of APE1 in oxidatively mediatated SIRT1 transcription. Under normal condition, APE1, together with other protein factors, is bound to nCaRE element present within SIRT1 promoter determining the basal activation of SIRT1 transcription. Conversely, upon oxidative stress conditions, DNA oxidation determines the formation of 8-oxodeoxyguanine (8-oxoG) lesions at nCaRE sequence present in SIRT1 promoter, which are recognized and processed by BER enzymes including APE1. The nicks introduced at the chromatin level by APE1, during 8-oxoG removal, might promote the formation of chromatin loops moving move closer the active form of RNA polymerase II to the TSS of the gene turning on the transcription.

Table 1. Dissociation constant and kinetic parameter values as determined for APE1 by SPR analysis.

Ligand	Ka (Ms 10^5)	Kd (1/s)	K_D (µM)
SIRT1-B nCaRE	0.270	0.105	3.90±0.08
SIRT1-B mutated	0.0198	0.236	119±8
polyT	0.004	0.112	308±3

Figure 1

A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8724
genes with
nCaRE
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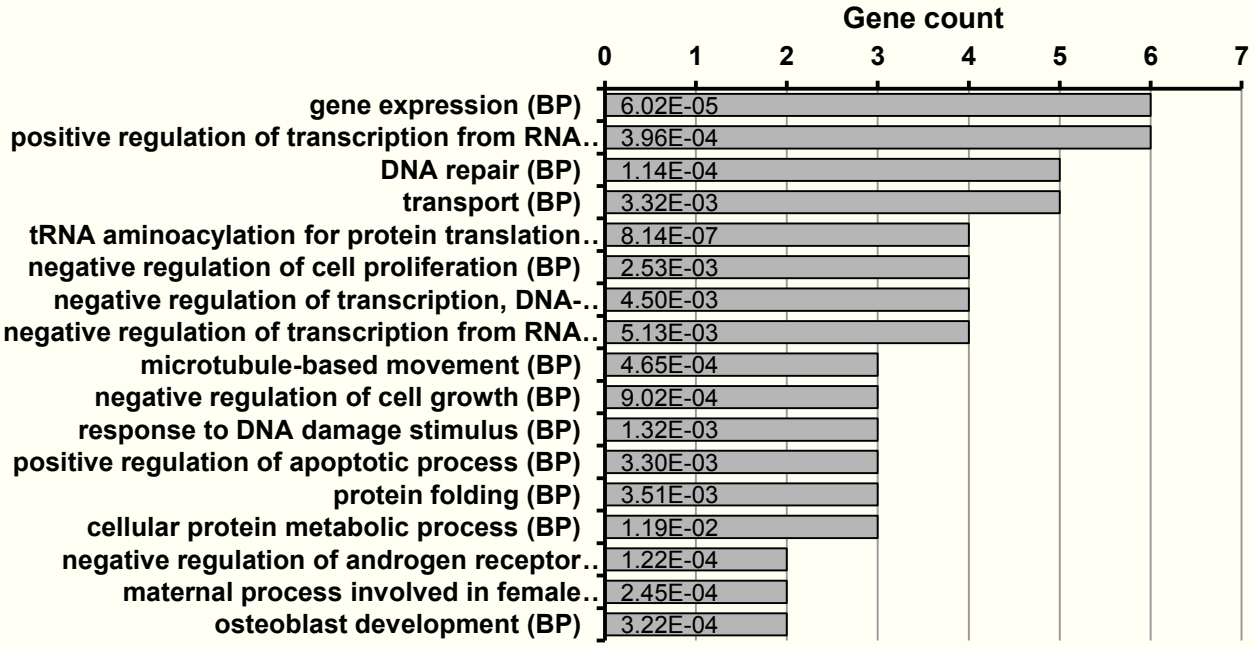
filter
8724
genes with
nCaRE
384
842
genes up/down
regulated
in microarray
384
genes with
nCaRE and
in microarray
profile
369
genes with
nCaRE
and
significant
GO terms
57
2137
mouse orthologous
genes with nCaRE

B


Gene count
0
1
2
3
4
5
6
7
gene expression (BP)
positive regulation of transcription from RNA..
DNA repair (BP)
transport (BP)
tRNA aminoacylation for protein translation..
negative regulation of cell proliferation (BP)
negative regulation of transcription, DNA-..
negative regulation of transcription from RNA..
microtubule-based movement (BP)
negative regulation of cell growth (BP)
response to DNA damage stimulus (BP)
positive regulation of apoptotic process (BP)
protein folding (BP)
cellular protein metabolic process (BP)
negative regulation of androgen receptor..
maternal process involved in female..
osteoblast development (BP)
6.02E-05
3.96E-04
1.14E-04
3.32E-03
8.14E-07
2.53E-03
4.50E-03
5.13E-03
4.65E-04
9.02E-04
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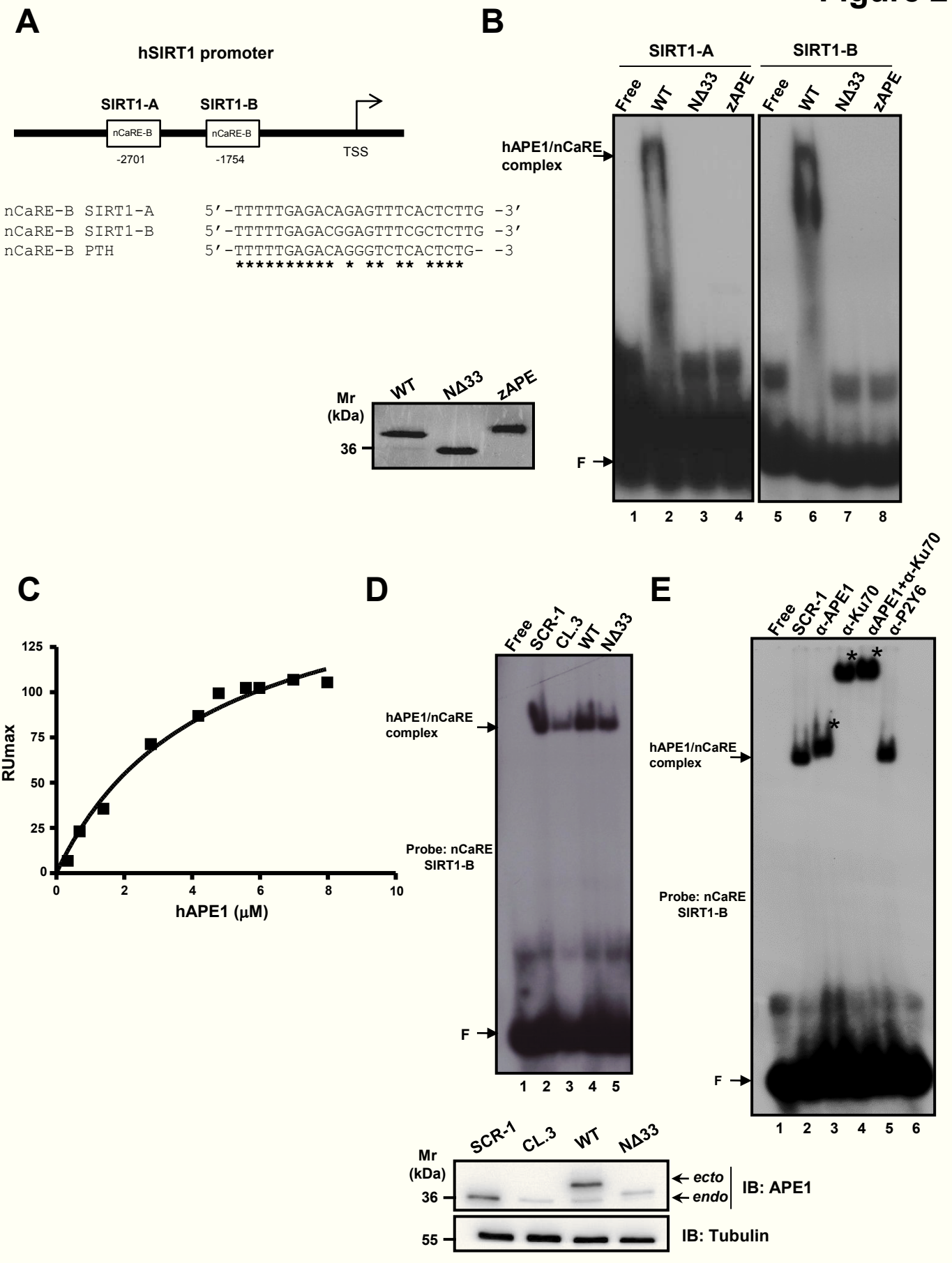
1.22E-04
2.45E-04
3.22E-04

Figure 2


A
hSIRT1 promoter
SIRT1-A
SIRT1-B
nCaRE-B
nCaRE-B
-2701
-1754
TSS
nCaRE-B SIRT1-A 5′-TTTTTGAGACAGAGTTTCACTCTTG -3′
nCaRE-B SIRT1-B 5′-TTTTTGAGACGGAGTTTCGCTCTTG -3′
nCaRE-B PTH 5′-TTTTTGAGACAGGGTCTCACTCTG- -3
********** * ** ** ****
B
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zAPE
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36
WT
NΔ33
zAPE
C
RUmax
125
100
75
50
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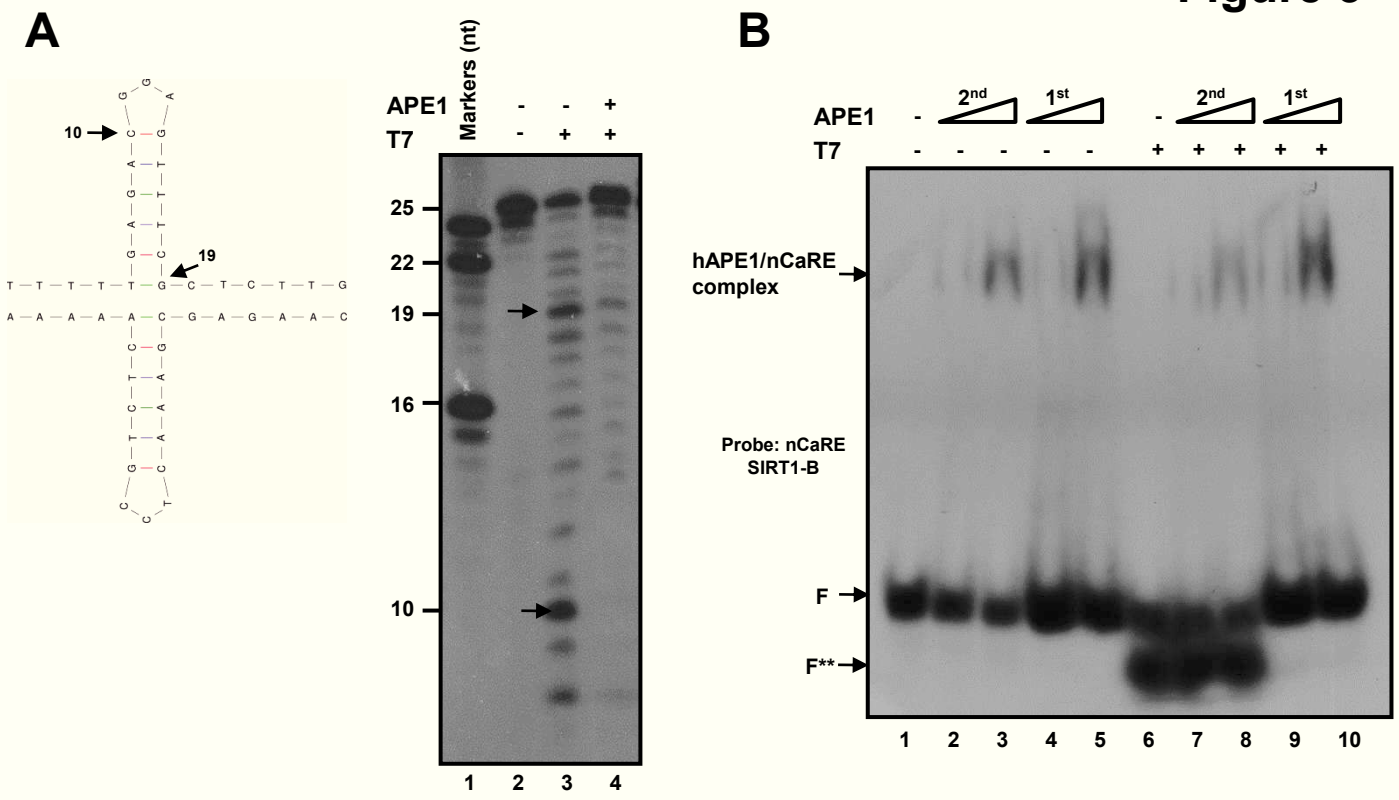
Free
SCR-1
CL.3
WT
NΔ33
hAPE1/nCaRE complex
Probe: nCaRE SIRT1-B
F
1 2 3 4 5
Mr (kDa)
36
55
SCR-1
CL.3
WT
NΔ33
ecto
endo
IB: APE1
IB: Tubulin
E
Free
SCR-1
α-APE1
α-Ku70
αAPE1+α-Ku70
α-P2Y6
hAPE1/nCaRE complex
Probe: nCaRE SIRT1-B
F
1 2 3 4 5 6

Figure 3


A
10
19
APE1
T7
Markers (nt)
25
22
19
16
10
1 2 3 4
B
APE1
2nd
1st
T7
hAPE1/nCaRE complex
Probe: nCaRE SIRT1-B
F
F**
1 2 3 4 5 6 7 8 9 10

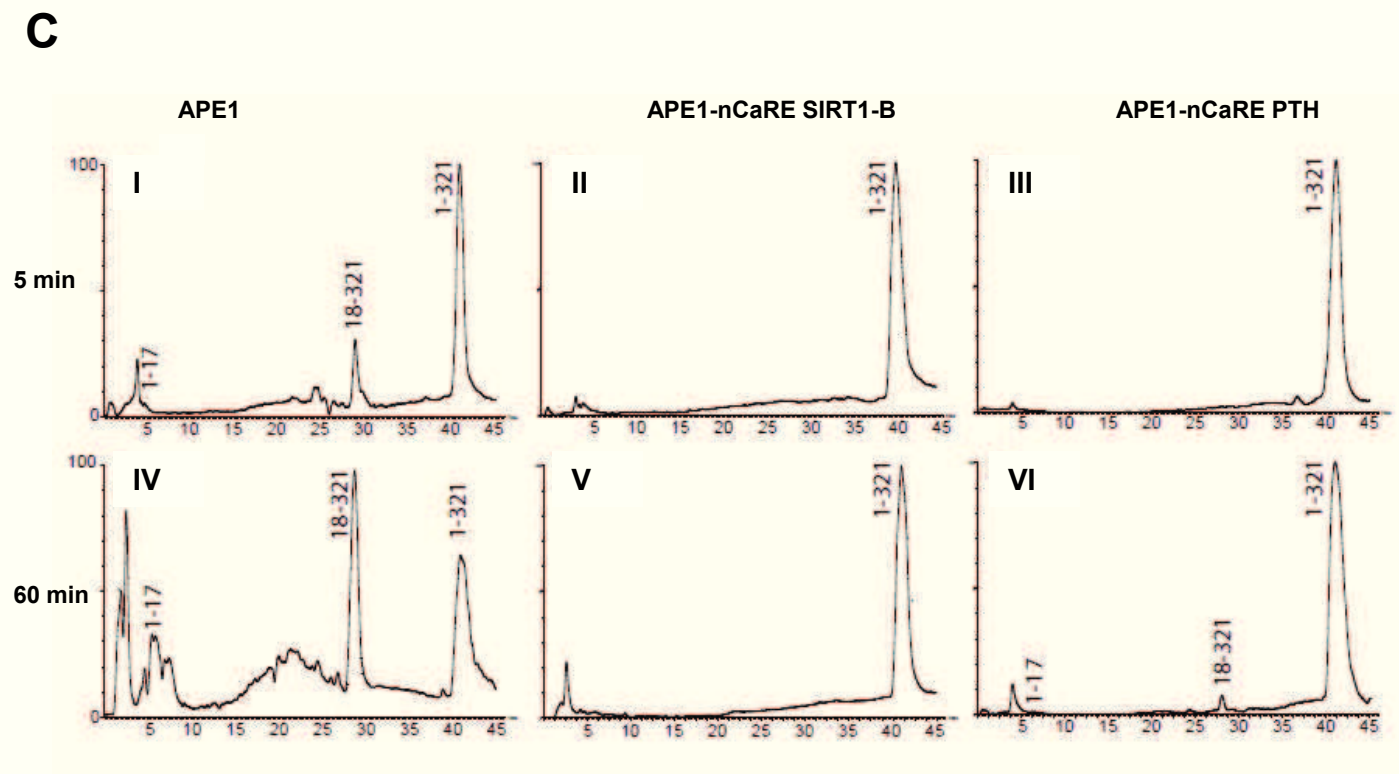

C
APE1
APE1-nCaRE SIRT1-B
APE1-nCaRE PTH
5 min
60 min
I
II
III
IV
V
VI
1-17
18-321
1-321

Figure 4

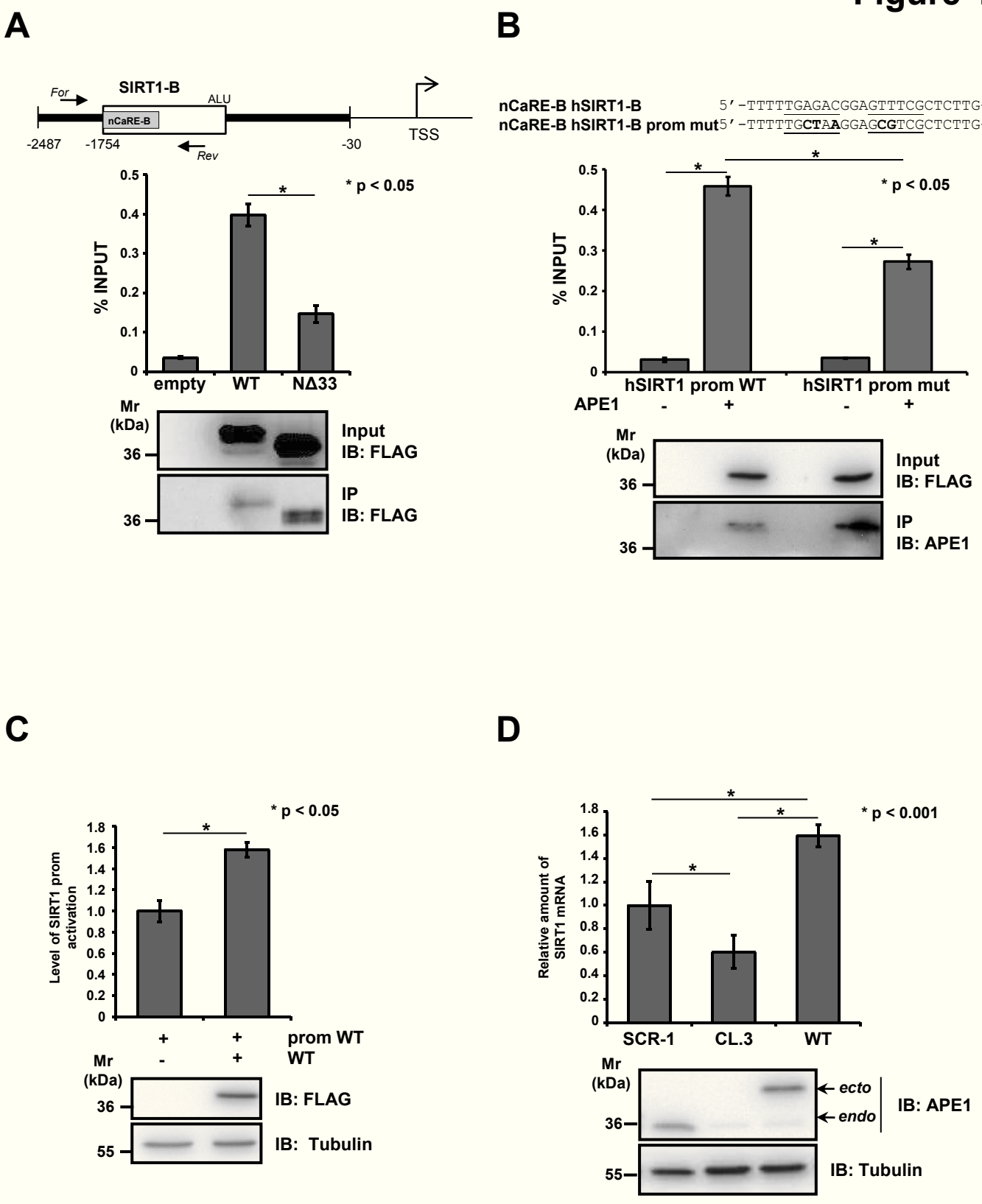

A
SIRT1-B
For
ALU
nCaRE-B
-2487
-1754
Rev
-30
TSS
* p < 0.05
% INPUT
empty
WT
NΔ33
Mr (kDa)
36
Input IB: FLAG
IP IB: FLAG
B
nCaRE-B hSIRT1-B 5′-TTTTTGAGACGGAGTTTCGCTCTTG-3′
nCaRE-B hSIRT1-B prom mut 5′-TTTTTGCTAAGGAGCGTCGCTCTTG-3′
* p < 0.05
% INPUT
hSIRT1 prom WT
hSIRT1 prom mut
APE1
Input IB: FLAG
IP IB: APE1
C
* p < 0.05
Level of SIRT1 prom activation
prom WT
WT
IB: FLAG
IB: Tubulin
55
D
* p < 0.001
Relative amount of SIRT1 mRNA
SCR-1
CL.3
WT
ecto
endo
IB: APE1
IB: Tubulin

Figure 5

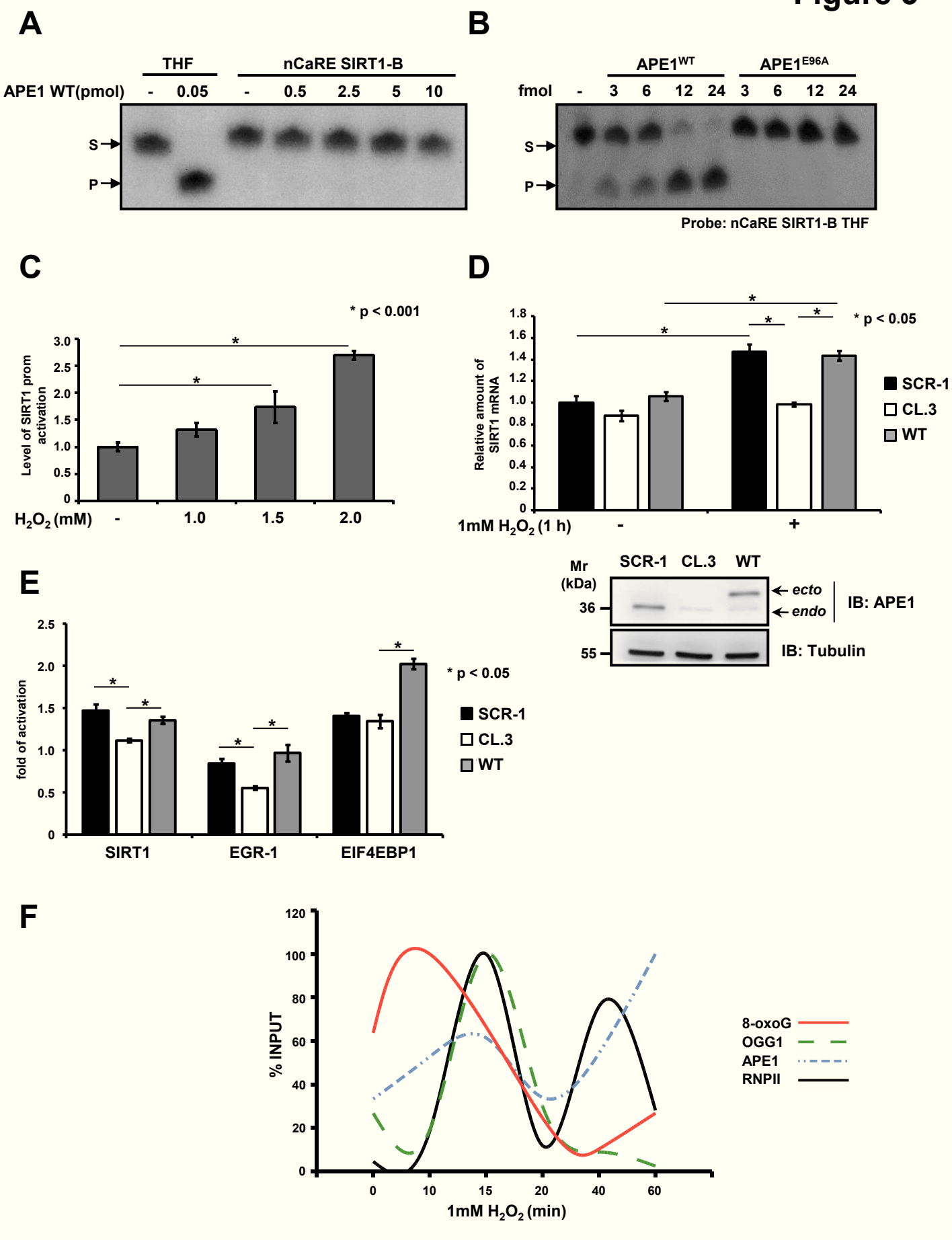
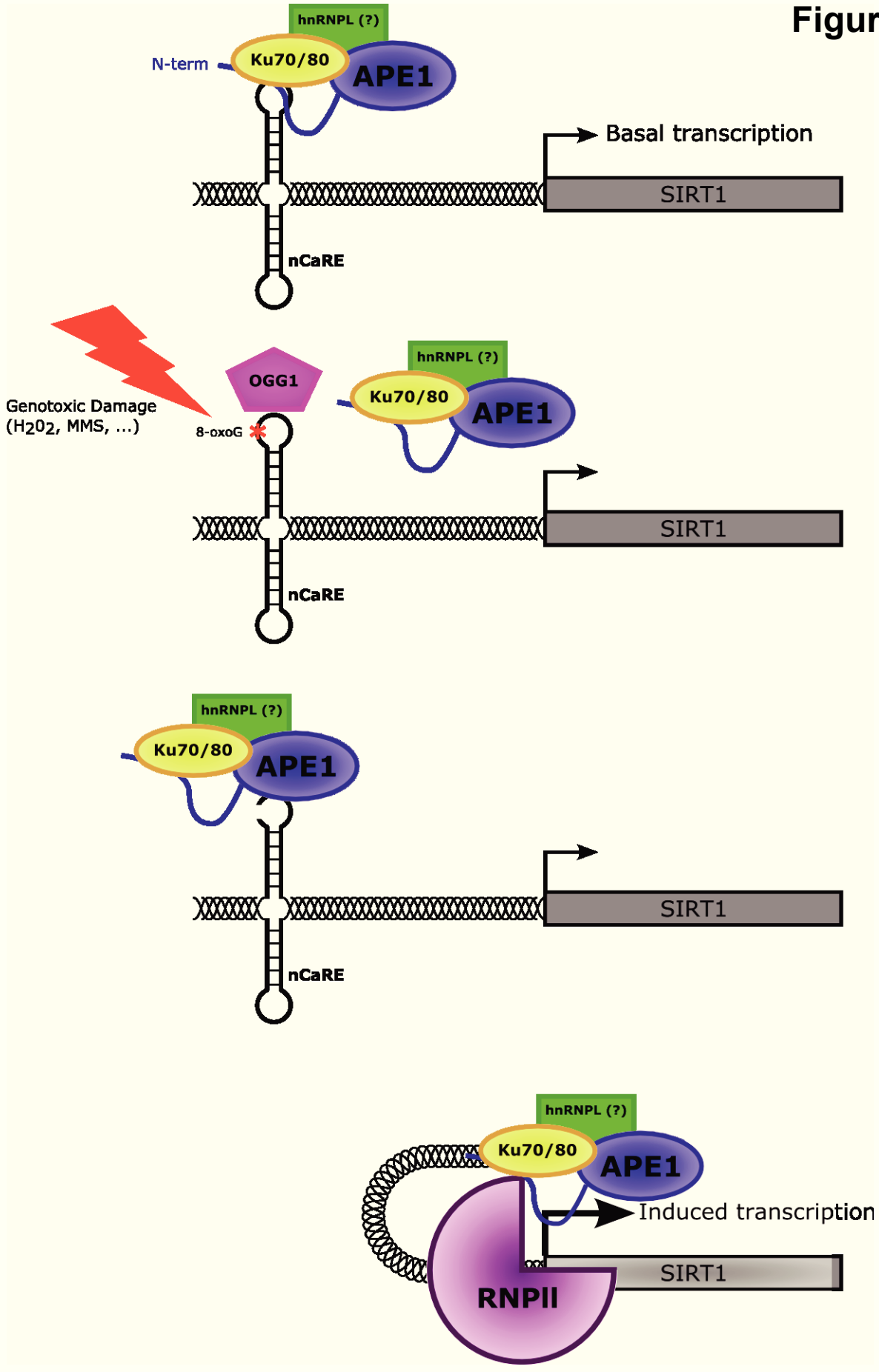

A
THF
nCaRE SIRT1-B
APE1 WT(pmol) - 0.05 - 0.5 2.5 5 10
S
P
B
APE1WT
APE1E96A
fmol - 3 6 12 24 3 6 12 24
S
P
Probe: nCaRE SIRT1-B THF
C
* p < 0.001
Level of SIRT1 prom activation
H2O2 (mM) - 1.0 1.5 2.0
D
* p < 0.05
Relative amount of SIRT1 mRNA
SCR-1
CL.3
WT
1mM H2O2 (1 h) - +
E
fold of activation
* p < 0.05
SCR-1
CL.3
WT
SIRT1
EGR-1
EIF4EBP1
Mr (kDa)
SCR-1 CL.3 WT
36
55
ecto
endo
IB: APE1
IB: Tubulin
F
% INPUT
8-oxoG
OGG1
APE1
RNPII
1mM H2O2 (min)

Figure 6


hnRNPL (?)
Ku70/80
APE1
N-term
Basal transcription
SIRT1
nCaRE
Genotoxic Damage
(H2O2, MMS, ...)
OGG1
8-oxoG
hnRNPL (?)
Ku70/80
APE1
SIRT1
nCaRE
hnRNPL (?)
Ku70/80
APE1
SIRT1
nCaRE
hnRNPL (?)
Ku70/80
APE1
Induced transcription
SIRT1
RNPII